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Clostridium difficile infection in neonatal piglets: Pathogenesis, risk factors, and prevention

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Clostridium difficile infection in neonatal piglets: Pathogenesis, risk factors, and prevention

by

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ABSTRACT

Clostridium difficile is a gram-positive, anaerobic, spore-forming bacterium and considered the major cause of antibiotic-associated diarrhea in many countries worldwide. *Clostridium difficile* infection (CDI) in humans causes pseudomembranous colitis. Clinical signs range from mild diarrhea to potentially toxic megacolon, bowel perforation, peritonitis and even death. Naturally occurring *C. difficile* infection has also been described in several non-human species including pigs, horses, primates, rabbits, rats, dogs and cats. The majority of cases, both humans and animals, are associated with disequilibrium of commensal intestinal microbiota which is often attributed to antibiotic treatments. The pathogenesis of CDI is tightly associated with the cellular effects of toxin A and toxin B and the immunologic response associated with these toxins. Despite the importance of these toxins, the specific role of each toxin on the pathophysiology of disease is not yet completely understood.

Clostridium difficile is one of the major causes of enteric disease in neonate piglets, yet risk factors associated with *C. difficile* infection in piglets are unknown. Furthermore, there is a general lack of prevention strategies available for swine medicine. In our first study we used snatch farrowed neonatal pigs to investigate the role of different inoculum doses, antimicrobial therapy, and piglet-age on the development of disease. Our results indicated that *C. difficile* dosage appears to be an important risk factor for CDI; 10 day-old pigs can develop disease associated with *C. difficile*, and antibiotic administration following inoculation is not a major contributor for disease in neonatal piglets. In our second study we investigated the use of lactobacillus and a non-toxigenic *C. difficile* strain (NTCD) as probiotics to prevent CDI in piglets. Our results showed that NTCD reduces the incidence of

CDI in piglets. Toxin levels, mesocolonic edema and histopathologic lesions were reduced when compared to positive control piglets. Usage of *Lactobacillus* sp. did not reveal any clear benefits. Lastly, we developed a gut-loop ligate model in 7 day-old piglets to study the pathophysiology of toxin A and B alone or in combination in different segments of small intestine and colon. Results demonstrated that the porcine intestinal loop model has the potential to become a valuable resource to further investigate the pathophysiology and associated-inflammatory response associated with individual toxins within different segments of the intestines. Also, there was a synergistic effect when toxins were administered simultaneously. Loops treated with toxin A only had slightly higher histologic scores when compared to toxin B alone.

CHAPTER 1. GENERAL INTRODUCTION

Introduction

Clostridium difficile is a gram-positive, strictly anaerobic, spore-forming bacillus first isolated by Ivan C. Hall and Elisabeth O`Toole at Colombia University in 1935. The bacterium was cultured from healthy neonates` stools, and was initially considered part of the normal fecal flora (Hall I. C., O`Toole, E. 1935). It was not until the 1970`s when Bartlett and others suggested that this bacterium was the cause of antimicrobial associated diarrhea (Bartlett J. G. *et al.,* 1977). Since then, *C. difficile* has become one of the most important enteric pathogens and the leading cause of antibiotic associate diarrhea worldwide. Recent reports from the Center for Disease Control and Prevention (CDC) showed *Clostridium difficile* infection (CDI) is responsible for approximately 14,000 annual deaths in the Unites States and healthcare costs between $436 million to $3.2 billion annually (Dubberke E. R. *et al.,* 2008; European Center for Disease Prevention and Control 2013; Gabriel L., Beriot-Mathiot, A. 2014; O'Brien J. A. *et al.,* 2007).

Clostridium difficile infection has been described in humans, pigs, horses, non-humans primates, rabbits, rats, dogs, hamsters and cats (Arroyo L. G. *et al.,* 2005; Debast S. B. *et al.,* 2009; Hopman N. E. *et al.,* 2011; Keessen E. C. *et al.,* 2011a; Norman K. N. *et al.,* 2009). The vast majority of cases are associated with disruption of the intestinal microbiota as may be commonly observed with antibiotic treatment or in neonatal animals with undeveloped microbiota (Lawley T. D. *et al.,* 2009; Rupnik M. *et al.,* 2009a).

Exotoxins A and B (TcdA and TcdB, respectively) are considered the major virulence factors associated with disease (Borriello S. P. 1998; Carter G. P. *et al.,* 2010; Davies A. H.

et al., 2011; Modi N. *et al.*, 2011; Voth D. E., Ballard, J. D. 2005). Additionally, some strains of *C. difficile* produce an ADP-ribosylating binary toxin; however, the role of this toxin in the pathogenesis of disease development has not been elucidated (Davies A. H. *et al.*, 2011). Toxin A and B are part of the large clostridia glucosylating toxin family with molecular masses of 308 and 250 kDa, respectively. Genes responsible for toxin production are located in a 19.6Kb locus, known as *C. difficile* pathogenic island (PaLoc). Toxins enzymatic domains will target Rho GTPases which include Rho, Ras and Cdc42. These molecules are involved in cellular signaling and cytoskeleton regulation and inactivation of these pathways eventually leads to cell rounding and death. (Chumbler N. M. *et al.*, 2012; Davies A. H. *et al.*, 2011; Dillon S. T. *et al.*, 1995; Jank T., Aktories, K. 2008; Just I. *et al.*, 1994; Keel M. K., Songer, J. G. 2007).

Clostridium difficile is also an important enteric pathogens in pigs during the first week-of-life. Songer and others have shown that in *C. difficile* affected porcine herds, up to two-thirds of the litters can be diseased, and within the litter the morbidity can be as high as 97-100% (Anderson M. A., Songer, J. G. 2008; Songer J. G. 2004). Mortality rates can vary significantly; however, mortality as high as 16% have been reported (Anderson M. A., Songer, J. G. 2008). Common gross and histologic lesions associated with CDI in piglets include mesocolonic edema and purulent ulcerative colitis, respectively.

Although the awareness of this disease has increased in swine production over the last decade, more research is needed to better understand basic principles such as risk factors, prevention, epidemiology and treatment.

Specific Aims

The overall objective of the studies described herein was to 1) investigate the potential risk factors associated with CDI in piglets 2) evaluate preventative alternatives for CDI in piglets and 3) develop of a *C. difficile in vivo* porcine ligated intestinal-loop model to investigate the role of toxins A and B in the pathogenesis and immunologic response of disease.

Risk factors, associated with CDI neonate piglets, evaluated in the first study included 1) different bacterial challenge doses ($2x10^3$, $2x10^6$, $2x10^9$ heat-activated *Clostridium difficile* spores) 2) prior antimicrobial therapy with Lincomycin (Lincocin®, Pfizer Animal Health, New York, NY), Ceftiour (Excede®, Pfizer Animal Health, New York, NY), Tylosin (Tylan®, Elanco Animal Health, Greenfield, IN) and Tulathromycin (Draxxin®, Pfizer Animal Health, New York, NY) , and 3) piglet age (10 days-old piglets were challenged).

The second study focused on the evaluation of the use of *Lactobacillus sp.* and a non-toxigenic *C. difficile* strain (NTCD) as probiotic alternatives to prevent the development of CDI in piglets. The final study`s objective was to develop a porcine ligated intestinal-loop model to investigate the role of toxins A and B and the cytokine-profile associated with *C. difficile* disease in different segments of the small intestine and colon.

Dissertation Organization

This dissertation is prepared in an alternate manuscript format. The dissertation is composed of six chapters and includes a general introduction, a literature review, three separate scientific manuscripts, and a general conclusion. References cited in the general introduction, literature review, and the general conclusion chapters are listed at the end of the dissertation. One manuscript has been published and two manuscripts have been submitted to

refereed scientific journals. The Ph.D. candidate, Paulo Arruda is the primary author of the manuscripts and is the principal investigator for the experimental work described.

The first manuscript describes the investigation of potential risk factors associated with the development of *C. difficile* disease in neonate piglets. This manuscript was published in the journal *Anaerobe*. The second manuscript describes the investigation of potential probiotics including non-toxigenic *C. difficile* and *Lactobacillus* sp. on the prevention of *C. difficile* disease in neonate piglets. This manuscript was submitted to *Preventative Veterinary Medicine*. The third manuscript describes the development of a porcine ligated intestinal-loop model to study the effects of *C. difficile* toxin A and B on different sections of small and large intestines. This manuscript has been submitted to the *Journal of Comparative Pathology*.

Literature Review

Historical background

Clostridium difficile was first isolated by Ivan C. Hall and Elisabeth O`Toole at Colombia University in 1935 from healthy neonates` stools, and was initially considered part of the normal fecal flora (Hall I. C., O`Toole, E., 1935). Interestingly, the bacterium was first named *Bacillus difficilis* due to the bacilli morphology and the difficulty associated with the isolation and culture of the bacteria; the word *difficilis* originated from Latin and translates to "difficult" in English. First attempts to associate the bacteria with a disease were made in 1962, at the time however, there was not enough scientific evidence to support the role of *C. difficile* as a primary pathogen (Smith L., King, E., 1962).

In the 1950's, coinciding with the beginning of the antibiotic-era, cases of antibiotic-associated diarrhea (AAD) started to become more prevalent. At that time *Staphylococcus*

aureus was believed to be the primary pathogen associated with such cases (Pearce C., Dineen, P., 1960). *Clostridium difficile* was not classified as a pathogen until the late 1970's when a small cohort of patients suffering from AAD was selected based on a common history of clindamycin administration. Conventional medical knowledge was challenged when bacterial culture from these patients failed to identify any known enteric bacterial pathogens. In addition, *S. aureus* was highly susceptible to clindamycin leading scientists to begin questioning the role of *S. aureus* in these cases (Tedesco F. J. *et al.,* 1974). In 1977, Bartlett and others isolated a clindamycin-resistant bacterium from the Clostridium family and, through hamster studies, suggested that this bacterium was the cause of AAD (Bartlett J. G. *et al.,* 1977). Later in 1977, two other research groups led by Larson and Price, and Rifkin *et al.* identified toxins in feces from affected and non-affected patients (Larson H. E., Price, A. B. 1977; Rifkin G. D. *et al.,* 1977). These finding were followed by the isolation of pure culture of *C. difficile* from affected patients and the identification of toxins. In 1978, the role of *C. difficile* was further investigated in naturally occurring cases and through the development of animal models (George R. H. *et al.,* 1978; George W. L. *et al.,* 1978). Since then, the bacterium has become one of the most important enteric pathogens and the leading cause of AAD worldwide. The bacterium is responsible for approximately 20% to 30% of all AAD reports in the United States (Cohen S. H. *et al.,* 2010; Gabriel L., Beriot-Mathiot, A., 2014).

Etiology

Clostridium difficile is an anaerobic gram-positive and spore-forming bacterium. Microscopically, the bacterium appears as short to long bacilli measuring approximately 3 to 5 microns in diameter. Optimum growth is achieved at 37°C, typically after 48 hours of

incubation, on blood agar plates. Classic colonies are described as flat, opaque to white, non-hemolytic, and irregular shaped. Colonies often fluoresce under UV light (Wren M., 2010) (Figure 1) and have a characteristic smell. Addition of taurochlorate acid is recommended as it facilitates sporulation and, therefore, yields improved culture results (Wren M., 2010). Treatment of fecal samples with alcohol at concentration of 50% prior to culture helps select *C. difficile* and controls other bacterial growth (Fedorko D. P., Williams, E. C., 1997). A selective media, cycloserine cefoxitin fructose egg yolk agar (CCFA) has been developed to facilitate the isolation of *C. difficile* (Bouza E. *et al.,* 2005; Brazier J. S., 1993).

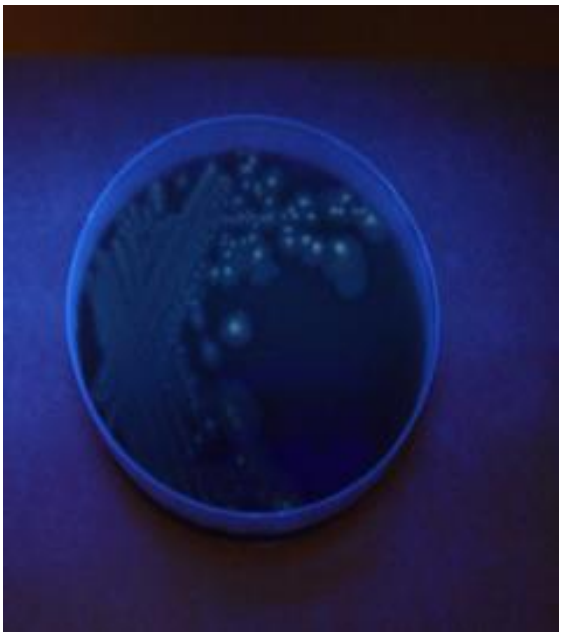


Figure 1. *C. difficile* colonies often fluorescence under UV light.

Clostridium difficile **infection**

Clostridium difficile infection has been described in diverse species of mammals including humans, pigs, horses, non-humans primates, rabbits, rats, domestic dogs, hamsters and domestic cats (Arroyo L. G. *et al.,* 2005; Debast S. B. *et al.,* 2009; Hopman N. E. *et al.,* 2011; Keessen E. C. *et al.,* 2011a; Norman K. N. *et al.,* 2009).The mammalian

gastrointestinal (GI) tract is colonized by high numbers of organisms including bacteria, viruses, and protozoans. Studies have shown that there are 10 times more commensal bacteria than somatic and stem cells in the human body (Backhed F. *et al.,* 2005; Hooper L. V. *et al.,* 2001; Hooper L. V., Gordon, J. I., 2001), an estimated 10^{14} commensal bacterial colonies (Ley R. E. *et al.,* 2006). In addition to the significant number of bacteria, the diversity of the bacterial population is extraordinary, making the GI tract the most densely populated microbial habitat known in biology (Gill S. R. *et al.,* 2006) and suggested by some scientists as one of the most complex ecosystems on the planet. A healthy human colon has been reported to contain more than 500 species (Artis D., 2008). A recent study investigating the pig intestinal microbiota revealed an even more diverse bacteria population composed of approximately 800 different species of bacteria (Looft T. *et al.,* 2012).

The complex and dynamic relationship among this vast and diverse bacteria population is highly associated with the development of several different diseases including *Clostridium difficile* infection (CDI). *Clostridium difficile* is also found within healthy individuals, in particular within neonates (Collignon A. *et al.,* 1993; Delmee M., 2001). An early study showed that 61% of healthy neonates are colonized (Holst E. *et al.,* 1981); however, only a significantly small proportion of them develop disease. Asymptomatic carriage by adults has also been described; however the prevalence is lower than neonates (Collignon A. *et al.,* 1993; Delmee M., 2001). Disruption of the normal intestinal flora is considered the most important risk factor associated with disease. This is best exemplified by the fact that the vast majority of CDI cases in humans are associated with antimicrobial therapy, making *C. difficile* one of the most important nosocomial disease in humans (Bartlett J. G. 2002; Carter G. P. *et al.,* 2010; Keel M. K., Songer, J. G. 2006; Kelly C. P.,

LaMont, J. T., 2008; McDonald L. C. *et al.,* 2005; Steele J. *et al.,* 2010). The same pattern is observed in animals; however, in addition to antimicrobial treatment, poorly developed intestinal flora in neonatal animals is also consider a major risk factor (Lawley T. D. *et al.,* 2009; Rupnik M. *et al.,* 2009b).

The bacterium is commonly found in the environment including soil, water, vegetables (al S. N., Brazier, J. S., 1996), and animals (Songer J. G., 1996). *Clostridium difficile* produces metabolically-dormant spores which play an essential role in bacterial survival when environmental conditions are harsh, and is directly associated with transmission, epidemiology and the pathogenesis of disease. *Clostridium difficile* spores are highly resistant to oxygen exposure, desiccation, and most common disinfectants, surviving for months and likely years in the environment (Wullt M. *et al.*, 2003). The transmission, for most species, is mostly fecal-oral and/or from the environment and primarily due to the characteristics mentioned previously. Spores can overcome the unhospitable acidic condition of the stomach (Nerandzic M. M. *et al.,* 2009) and, once in the large intestines and under proper micro-environmental conditions, are able to germinate and colonize.

Pathogenesis and toxin action

Numerous virulence factors associated with CDI have been discovered including surface proteins and exotoxins, though there is a consensus that exotoxins A and B (TcdA and TcdB, respectively) are the major virulence factors associated with disease development (Borriello S. P. 1998; Carter G. P. *et al.,* 2010; Davies A. H. *et al.,* 2011; Modi N. *et al.,* 2011; Voth D. E., Ballard, J. D., 2005). Some strains of *C. difficile* also produce an ADP-ribosylating binary toxin, however the role of this toxin on the pathogenesis of disease development is still not well understood (Davies A. H. *et al.,* 2011). Approximately 20% of

C. difficile strains are not capable of producing toxins and, therefore, considered nonpathogenic (Kelly C. P. *et al.,* 1994).

Toxins A and B have enterotoxic and cytotoxic affects respectively (Taylor N. S. *et al.,* 1981). Toxin A also possesses cytotoxic affects but is 100-fold less potent than TcdB (Donta S. T. *et al.,* 1982). It was previously thought that TcdA alone was responsible for most of the clinical signs and pathology associated with CDI, and TcdB was not toxic to animals unless TcdA was administered simultaneously (Lyerly D. M. *et al.,* 1985; Lyras D. *et al.,* 2009). Recent studies and the appearance of the 027 ribotype strain (characterization of this ribotype revealed that this bacterium is $TcdA^-$, $TcdB^+$ and highly pathogenic) have changed the way we understand the pathogenesis of this disease. Genetically modified strains of *C. difficile*, created through truncation and subsequent inactivation of particular toxin genes, have allowed the investigation of specific toxin roles in hamster and tissue culture models. Results from these studies showed that TcdB, not TcdA, is essential for disease development; furthermore, disruption of TcdB genes significantly reduces the virulence phenotype (Lyras D. *et al.,* 2009).

Toxins A and B are part of the large clostridia glucosylating toxin family with molecular masses of 308 and 270 kDa, 2,710 and 2,366 amino acids, respectively, making these toxins two of the largest bacterial toxins that have been characterized. The amino acid sequences of TcdA and TcdB are 49% identical and 63% to 66% similar. The genes responsible for toxin production are located in a 19.6Kb locus, known as *C. difficile* pathogenic island (PaLoc). The genes responsible for regulation of toxin production are also located within the PaLoc (Davies A. H. *et al.,* 2011; Rupnik M. *et al.,* 2005; Tan K. S. *et al.,* 2001).

Toxin A and B are composed of a multi-domain structure that contain an N-terminal domain, a C-binding terminal domain, a cysteine protease domain, and a hydrophobic domain (Davies A. H. *et al.*, 2011; Jank T., Aktories, K., 2008). The C-binding terminal domain is involved in toxin binding to cell carbohydrate receptors (Chumbler N. M. *et al.*, 2012; Davies A. H. *et al.*, 2011; Keel M. K., Songer, J. G., 2007); however, the toxin segment varies significantly between TcdA and TcdB suggesting that different receptors on the cell surface are involved in binding the two toxins (Keel M. K., Songer, J. G., 2007). Alpha-galactosyl receptors with a Gal"1-4GlcNAc core were identified as receptors for TcdA in brush border surfaces of hamsters (Krivan H. C. *et al.*, 1986) and on human intestinal epithelial cells (Tucker K. D., Wilkins, T. D., 1991). A recent study conducted by Keel and Songer showed that the α-galactosyl receptors within piglet enterocytes are likely not significant for *C. difficile* toxin binding (Keel M. K., Songer, J. G., 2007). Cellular receptors for TcdB have not yet been identified (Voth D. E., Ballard, J. D., 2005). Lyerly and others showed that intact mucosal surfaces are resistant to the effects of TcdB, and prior trauma are likely necessary for toxin internalization (Lyerly D. M. *et al.*, 1985). This finding led scientists to the hypothesis that the receptor might be on the basolateral side of the cells (Keel M. K., Songer, J. G., 2007). Both toxins are internalized via receptor-mediated endocytosis. Subsequent to toxin internalization, a decrease in the pH within the endosome compartment results in a toxin structural change, exposure of the hydrophobic domains, and pore formation which allows the release of the enzymatic domain of the toxin (N-terminal) into the cell cytosol. Once within the cytosol of the cell, TcdA and TcdB target Rho GTPases which include Rho, Ras and Cdc42. These molecules are involved in cell signaling and actin cytoskeleton regulation. Inactivation of these molecules disrupts cell signaling and

compromises actin cytoskeleton regulation, eventually leading to cell rounding, detachment from basal membrane, and death (Chumbler N. M. *et al.*, 2012; Davies A. H. *et al.*, 2011; Dillon S. T. *et al.*, 1995; Jank T., Aktories, K. 2008; Just I. *et al.*, 1994; Keel M. K., Songer, J. G., 2007).

Enterocyte death and damage to the epithelial tight junctions leads to erosions and ulcerations, increased permeability, and a concomitant acute inflammatory process primarily characterized by a neutrophilic infiltration and variable amounts of fibrin and proteinaceous fluid (pseudomembrane colitis). Pro-inflammatory cytokines such as IL-1β, TNF-α and IL-8 are up-regulated within affected areas (Fiorentini C. *et al.*, 1998; Kelly C. P., Kyne, L., 2011; Mahida Y. R. *et al.*, 1996; Savidge T. C. *et al.*, 2003) and upon exposure to TcdA and prior to cytoskeleton damage, there is activation of NF-kB signaling within enterocytes and production of cytokines such as IL-8 (He D. *et al.*, 2002). Dan He and others have shown that this inflammatory pathway is independent of cytoskeleton damage (He D. *et al.*, 2002). It has also been shown that these toxins are able to induce apoptosis in epithelial cells, T-cells, and eosinophils (Fiorentini C. *et al.*, 1998; Mahida Y. R. *et al.*, 1996).

Clinical signs in humans

Clostridium difficile infection affects more than 3 million patients per year in the United States (Kelly C. P. *et al.*, 1994; McDonald L. C. *et al.*, 2006; Schroeder M. S., 2005; Starr J., 2005). The spectrum of disease is highly variable, from asymptomatic to mild self-limited diarrhea to more severe cases of colitis. Clinical signs in humans are primarily gastrointestinal, and typically involve the colon. Signs include differing levels of diarrhea, fever, abdominal pain, and dehydration (Starr J., 2005). Complications attributed with disease include intestinal perforation, toxic megacolon, and sepsis. Disease reoccurrence is

reported and often associated with drug-resistant strains and fulminant colitis resulting in the need for a colectomy. Death occurs in approximately 3% to 5% of patients (Sailhamer E. A. *et al.,* 2009).

Clostridium difficile animal models

Clostridium difficile was recognized as a cause of antimicrobial associated diarrhea (AAD) in the late 1970`s and animal models to investigate pathophysiology, bacterial colonization and ecology, population dynamics, risk factors and potential treatments have been under development and are still being refined (Best E. L. *et al.,* 2012). Several different animals have been used as models to study CDI; some examples include the hamster, mice, guinea pig, rat, prairie dogs, quails, rabbits and pigs (Best E. L. *et al.,* 2012; Lawley T. D., Young, V. B., 2013). The first documented hamster model dates back to 1968 (Small J. D. 1968); and currently, the hamster model is the most common animal model used to study CDI. Despite the availability of reagents and a large body of literature regarding this model, hamsters are exquisitely sensitive to the toxins' effects (after antibiotic administration) and commonly die within a few days (Best E. L. *et al.,* 2012; Keel M. K., Songer, J. G., 2006). The Rapid and uniformly fatal disease (Best E. L. *et al.,* 2012; Keel M. K., Songer, J. G., 2006; Lyerly D. M. *et al.,* 1985) differs significantly from CDI in humans. Furthermore, the rapid progression of disease in this species also poses obstacles when investigating possible treatment and/or prevention options. Chen and others (Chen X. *et al.,* 2008) have shown that genetically normal mice, when pretreated with a combination of antibiotics, can develop disease which in some circumstances leads to death; however; some of the signs appeared to be transient and recovery usually occurred within 4 days (Chen X. *et al.,* 2008; Spencer J. *et al.,* 2014).

The disease occurs naturally in swine. Pigs are moderately sensitive to the effects of *C. difficile* toxins and the severity of clinical signs and histologic lesions is often related to piglet-age and challenge dose (Steele J. *et al.,* 2010). Additionally, the marked similarities in nature and progression of CDI in pigs to human disease (Best E. L. *et al.,* 2012; Steele J. *et al.,* 2010) and the wide range of assays and immunoreagents commercially available make the pig model an attractive alternative to study the disease. Currently there are two piglet models suitable to study disease. The first model utilizes gnotobiotic piglets, caesarian-derived and housed inside sterile isolators for the duration of the study (Steele J. *et al.,* 2010). The second model developed by Lizer and others (Lizer J. T. *et al.,* 2012) used snatch-farrow commercial piglets housed in a biosecurity level-2 laboratory.

Clostridium difficile infection in neonatal piglets

The newborn piglet is born with a sterile gastrointestinal (GI) tract, however colonization by mixed populations of bacteria occur within hours of birth (Ducluzeau R., 1983; Mackie R., I *et al.,* 1999; Mackie R. I. *et al.,* 1999). These bacteria are mechanically acquired by piglets via oral contact with the dam's vaginal canal and perineum, exposure to the environment, exposure to feces, teat suckling, and skin contact (Mackie R., I *et al.,* 1999; Smith H., 1965). Studies investigating the first intestinal colonization of neonatal piglets have identified that *Lactobacilli sp, Streptococcus sp, Fusobacterium sp, Clostridium perfringens sp,* and *Escherichia coli* are among the first organisms to colonize the small intestine and colon (Pesti L. 1962; Smith H., Crabb, W., 1961; Wilbur R. *et al.,* 1960). Several factors are believed to play a role in the dynamic succession of microorganism colonization. Rapid and drastic changes occur within the intestinal environment after a piglet is born. For instance, the abrupt exposure of the GI tact to air and milk may significantly

shape the environment, and indirectly select for better fit animals. Within the first few minutes of life, microbes within the GI tract compete for niches in a process of succession that will eventually establish the intestinal flora. This process of colonization is incredibly complex, and likely influenced by the dam's flora, environmental bacterial population, antimicrobial usage, source and quantity of nourishment, modulated by acquired immunity from the dam (antibodies and immunological molecules in milk), and lastly modulated by active immune responses of the piglet (Ducluzeau R., 1983; Mackie R., I *et al.,* 1999).

Clostridium difficile intestinal colonization occurs within the first hours of life in the neonatal pig, and virtually one hundred percent of piglets in some herds are colonized within 48 h of birth (Hopman N. E. *et al.,* 2011). Despite the high percentage of colonization, not all piglets within a litter develop disease. Studies investigating affected herds have shown that on average two-thirds of the litters are clinically affected, and within the litter the morbidity can reach 97 to 100% (Anderson M. A., Songer, J. G., 2008; Songer J. G., 2004). Mortality rates associated with outbreaks of CDI is variable; however, some have reported mortality rates as high as 16% (Anderson M. A., Songer, J. G., 2008). Retardation of growth and lower weaning weights, approximately 0.5 Kg lighter on average, have been reported in recovered piglets (Songer J. G., 2004).

The prevalence of *C. difficile* carriage drops dramatically with age. Studies in a variety of countries investigating the prevalence of *C. difficile* in slaughter pigs revealed a consistently low prevalence, ranging from as low as 0% to approximately 8% (Baker A. A. *et al.,* 2010; Hoffer E. *et al.,* 2010; Keessen E. C. *et al.,* 2011c; Rodriguez C. *et al.,* 2013). Geographic locations, seasonality, and methodological differences are likely responsible for the variation described (Hensgens M. P. *et al.,* 2012; Rodriguez-Palacios A. *et al.,* 2009;

Weese J. S., 2010). Interestingly, Schneeberg and others have found that some ribotypes such as 078, 126 and 413/FLI01 are more likely to be found in pigs older than 2 weeks (Schneeberg A. *et al.,* 2012; Schneeberg A. *et al.,* 2013). Similar results have been reported in calves where ribotypes 078 and 126 appeared to be able to colonize longer than other ribotypes (Zidaric V. *et al.,* 2012). These results suggest that some ribotypes may be better adapted to their host species.

Epidemiologic studies investigating the presence of *C. difficile* in retailed meat showed that the bacteria can be found at low-levels in uncooked pork (Curry S. R. *et al.,* 2012). Similar results were found when other types of meat such as poultry, beef, veal, sea food, fish and vegetables were investigated (Harvey R. B. *et al.,* 2011; Houser B. A. *et al.,* 2012; Metcalf D. *et al.,* 2010; Metcalf D. *et al.,* 2011; Metcalf D. S. *et al.,* 2010; Rodriguez-Palacios A. *et al.,* 2007; Weese J. S. *et al.,* 2009; Weese J. S. *et al.,* 2010).

Lesions associated with disease are primarily concentrated in the large intestine. Macroscopic examination of affected animals reveals mesocolonic edema (Figure 2).

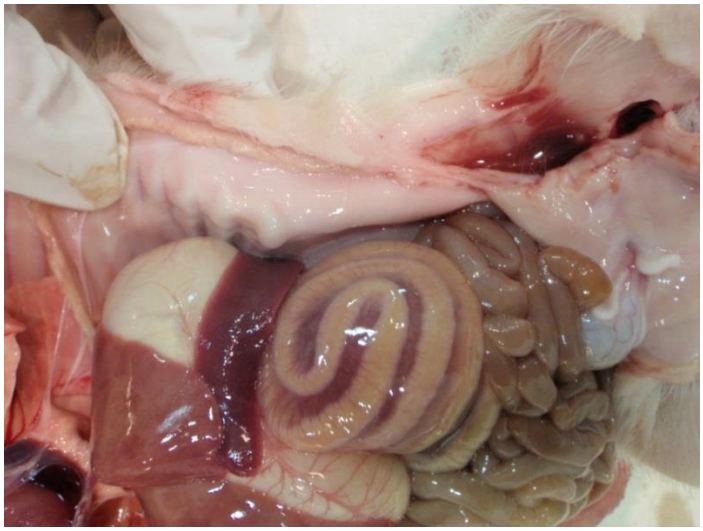


Figure 2. Severe mesocolonic edema observed in a 4-day-old piglet challenged with toxigenic *C. difficile.*

Microscopic examination reveals a multifocal to locally extensive ulcerative fibrinopurulent colitis. Gnotobiotic pigs have been described as having systemic disease resulting in ascites, pleural effusion, hepatic abscesses, renal dysfunction, and acute respiratory distress (Steele J. *et al.*, 2010). The mechanism by which the microorganism causes systemic disease is not yet completely understood.

In contrast to CDI in humans, where old-age is a risk factor, the disease in swine is predominately observed within 1-7 day-old piglets (Hopman N. E. *et al.*, 2011; Songer J. G., Anderson, M. A., 2006). Neonatal piglets are highly susceptible to *C. difficile* colonization as the intestinal microflora is not fully established. A recent study conducted by Arruda and others (Arruda P. H. *et al.*, 2013) demonstrated that 10 days-old piglets are as susceptible as neonatal piglets when challenged with a toxigenic strain of *C. difficile*. In this particular study, piglets were kept in a research environment from birth to the day of challenge, and therefore may not be colonized by the microbiota present in a typical farm environment. This likely played a role on the susceptibility to disease of these older animals.

Many risk factors are thought to contribute to CDI including: administration of antimicrobials, challenge dose, associated toxin-profile, and animal age. *Clostridium difficile* is the major cause of AAD in humans (Bartlett J. G. 2002; Carter G. P. *et al.*, 2010; Keel M. K., Songer, J. G., 2006; Kelly C. P., LaMont, J. T., 2008; McDonald L. C. *et al.*, 2005; Steele J. *et al.*, 2010), however in pigs, scientific evidence for the role of antibiotic usage and development of disease is lacking (Waters E. H. *et al.*,1998; Yaeger M. *et al.*,2002; Yaeger M. J. *et al.*, 2007). A recent study investigated the role of antibiotic usage on neonatal piglets in the development of CDI (Arruda P. H. *et al.*, 2013). Results from this study did not show

any statistical association between antimicrobial usage and disease development or severity (Arruda P. H. *et al.*, 2013).

Disease diagnosis in piglets

There is a significant gap in knowledge regarding CDI diagnosis in animals, and there are no official guidelines available for *C. difficile* diagnosis. The endemic nature and ecological characteristics of this bacterium raise significant challenges for diagnosis. For instance, simple culture and isolation of the organism should not be used solely as a diagnostic test given the organism can be isolate from healthy animals. Commercially available molecular diagnostics, such as real time PCR targeting the detection of the TcdB gene (Crobach M. J. *et al.*, 2009), should not be used as a sole diagnostic since this test falls for the same reason that mere identification of the organism is not sufficient to diagnose disease.

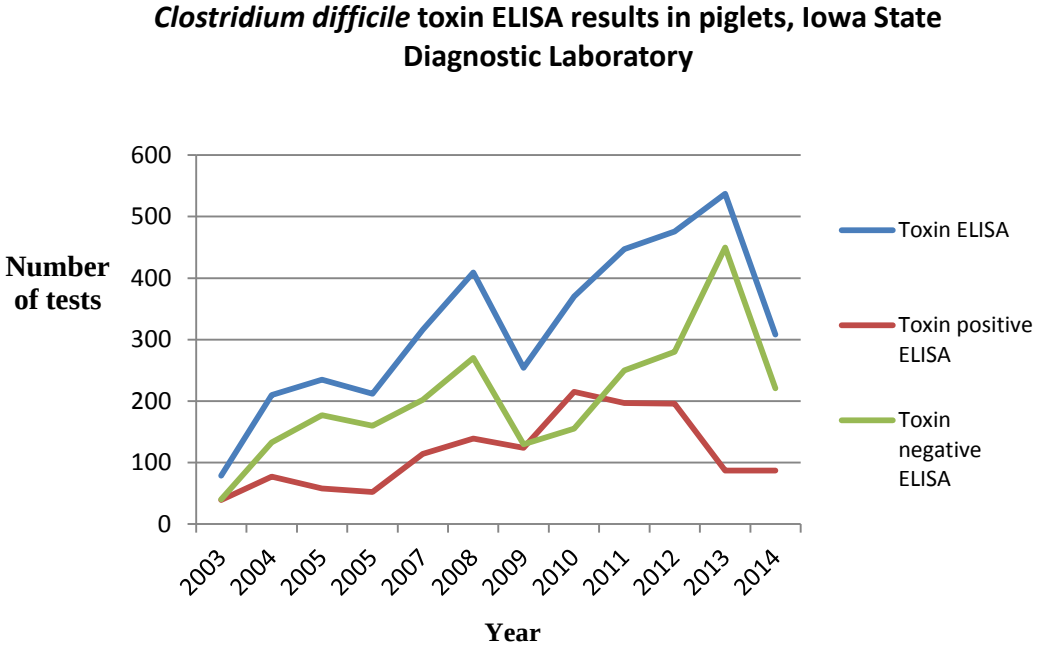
Identification of an age-group risk (1-7 days-old piglets), and systematic examination of clinical signs (watery yellow diarrhea) are the first steps to identify potential cases. At necropsy, macroscopic examination of the carcass can reveal mild to severe mesocolonic edema (Figure 2). However, mesocolonic edema in piglets is not a good predictor of *C. difficile* toxins (Yaeger M. J. *et al.*, 2007). Though this lesion is suggestive of disease, it is not pathognomonic of CDI.

Clinical signs are primarily due to the release of exotoxins, in this case TcdA and TcdB. Cell culture cytotoxicity neutralization assays is considered the gold standard for toxin detection (Bartlett J. G., 2006); however, this test is time-consuming, taking approximately 1-2 days, and requires access and expertise with cell culture methods. There are currently different commercial enzyme immunoassays (ELISA assays) targeting TcdA and TcdB.

These tests were developed and extensively evaluated for use in human infections and have not been extensively evaluated for use in swine. Studies investigating the sensitivity and specificity of two commercial ELISA kits Tox A/B II (Techlab, Blacksburg, VA) and Gastro-Tect *Clostridium difficile* Toxin A+B (Medical Chemical Corp, Torrance, CA) on piglet feces revealed a much lower sensitivity and specificity when compared to human stools (Keessen E. C. *et al.,* 2011b). Other studies evaluating the use of ELISA assays on stools from horses, pigs and dogs showed similar results where sensitivity and specificity were significantly lower when compared to human cases (Anderson M. A., Songer, J. G., 2008; Arroyo L. G. *et al.,* 2007; Chouicha N., Marks, S. L., 2006). Stools and/or colonic content from diarrheic piglets are the recommend sample to perform the ELISA assays despite lower sensitivity and specificity in comparison to human samples (Keessen E. C. *et al.,* 2011b).

In addition to complexity and flaws in the toxin-detection tests, Yaeger and others showed that when additional enteric pathogens were excluded, pigs with toxin-positive results were significantly more likely to have normal feces when compared to toxin-negative pigs (Yaeger M. J. *et al.,* 2007). The same study showed that a high percent of apparently healthy pigs were positive for the toxins. *Clostridium difficile* toxin ELISA is routinely used for *C. difficile* diagnosis at the Iowa State Diagnostic laboratory. Table below summarizes the total number of *C. difficile* ELISA(s) performed annually at the Iowa State Diagnostic Laboratory (ISU-VDL) and the number of negative and positive results respectively.

Table 1. Total number of *Clostridium difficile* ELISA tests performed at the ISU-VDL per year since 2003 in addition to the total number of positive and negative results.



At necropsy, a longitudinal section including several colonic loops should be placed in 10% formalin for histopathologic examination. Microscopic lesions observed are characterized by variably neutrophilic infiltration admixed with low to abundant amounts of fibrin and cellular and karyorrhectic debris. Inflammatory exudate is often associated with multifocal to coalescing areas of erosion and ulceration, also known as volcano-like lesions (Figure 4.) (Songer J. G., Uzal, F. A., 2005). Histologic lesions are highly suggestive of disease, however not pathognomonic. Yaeger and other have shown that histologic lesions such as erosion, ulcerations and a neutrophilic infiltration are good predictor of the presence of the toxins (predict value of 76%)

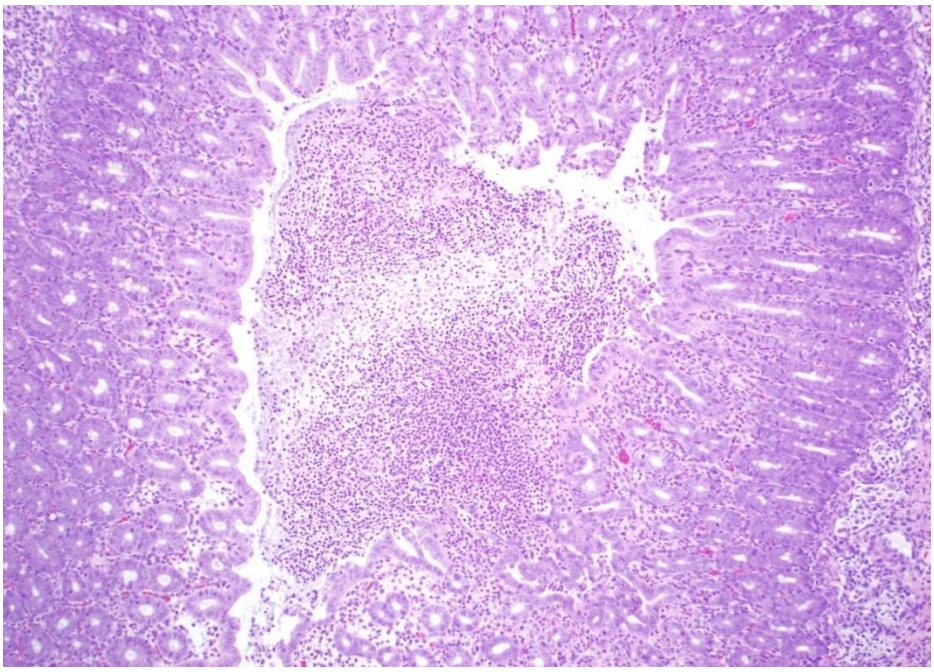


Figure 4. Histopathologic examination of a piglet challenged with toxigenic *C. difficile.* Section of colon: classic volcano-like lesion characterized by a focal ulceration and replacement by moderate to abundant amounts of cellular and karyorrhectic debris, degenerate neutrophils, and fibrin. Additionally, expanding lamina propria and separating crypts are neutrophils and cellular and eosinophilic debris.

At the ISU-VDL a multi-step diagnosis process including the identification of potential cases (clinical history), toxin-detecting ELISA assays, and histologic examination is recommend to properly diagnose CDI in piglets. Summary of the ISU-VDL diagnosed data revealed that approximately 8984 cases of enteric disease in pigs within the first-week-of life were diagnosed since 2003 to the present; *C. difficile* was diagnosed in 501 of the cases. Figure below summarizes the major factors associated with *C. difficile* in piglets including aspects associated with epidemiology, transmission and pathogenesis of disease.

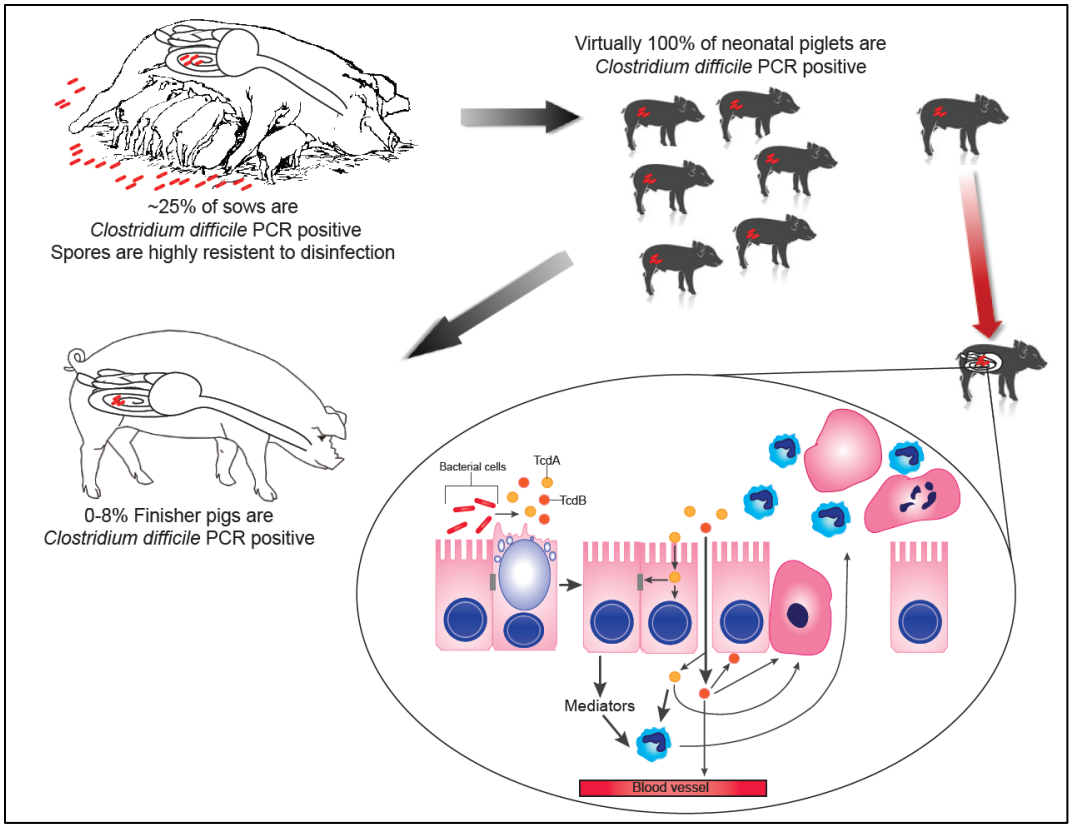


Figure 5. Clostridium difficile intestinal colonization occurs within the first hours of life in the neonatal pig, and virtually one hundred percent of piglets in some herds are colonized within 48 h of birth. Majority of spores are present with environment and a smaller percentage within sow`s gastrointestinal tract. Despite the high percentage of neonatal colonization, not all piglets within a litter develop disease. *Clostridium difficile* colonizes the colonic epithelium and produces toxins A (TcdA) and B (TcdB). Toxin A binds to receptors within the apical border of enterocyte and is internalized via receptor-mediated endocytosis. Disruption of tight junctions and cell rounding allows toxin B to bind to lateral base border of cells. Toxin A and B are involved in cell signaling and actin cytoskeleton regulation and can eventually reach blood vessels within submucosa and in combination with cytokine produced by enterocyte death attract neutrophils to affected area. The prevalence of *C. difficile* carriage animals drops dramatically with age. Studies in a variety of countries investigating the prevalence of *C. difficile* in slaughter pigs revealed a consistently low prevalence, ranging from as low as 0% to approximately 8%.

Alternatives for prevention of *Clostridium difficile* infection in neonatal piglets

In humans, the prevention of *C. difficile* disease is primarily divided in two major categories: first minimize and/or avoid disruptions of the normal intestinal flora, and

secondly decrease the exposure of susceptible patients to the bacteria or spores. Similar principles apply to the disease in piglets; however, the disease is primarily observed in neonatal piglets likely due to lack of an established intestinal flora. To date, there are no commercially available products to prevent and/or treat CDI in piglets. Results from a recent study investigating possible risk factors in piglets show that development and severity of lesions can be associated with challenge dose, and therefore alternatives to decrease the exposure-dose to neonatal piglets might prevent and/or minimize disease (Arruda P. H. *et al.*, 2013). The process of cleaning and disinfection is particularly complicated due to the fact that *C. difficile* spores are highly resistant to commonly used disinfectants (Wullt M. *et al.*, 2003).

The use of immunotherapy is also considered a good strategy to prevent and control disease. In 1991, Lyerly and others investigated the use of antibodies from previously immunized cows to prevent disease in hamsters challenged with CDI. Results from this study revealed that study-hamsters were protected against the effects of the toxins (Lyerly D. M. *et al.*, 1991). More studies utilizing different sources of antibodies followed this study, for instance, hamsters were protected from lethal challenge with CDI when previously administered avian immunoglobulins against TcdA and TcdB (Kink J. A., Williams, J. A., 1998). Positive results from these studies and others encouraged the further test in humans. To date, there were a total of 15 small clinical trials, and results from these trials suggest that intravenous administration of antibodies directed against toxins is beneficial to patients suffering from CDI (Leung D. Y. *et al.*, 1991; Rebeaud F., Bachmann, M. F., 2012). Along the same lines, researchers investigated the used of equine-originating antitoxins to prevent disease in piglets. Results from this study show a statistical benefit of this technique.

Immunoglobulins were not able to completely prevent disease, but it significantly decreased the severity of histologic lesions associated with CDI on neonatal piglets (Ramirez A. *et al.*, 2014). More studies are necessary to understand the real benefit and the possible logistical issues associated with the use of such techniques in modern swine production.

Disruption of the intestinal flora and/or lack of flora are considered to be the major risk factors and restoration is important for the recovery from CDI. Different studies have shown that normal GI flora inhibits *C. difficile* growth and toxin release (Borriello S. P., 1990; Parkes G. C. *et al.*, 2009). According to the World Health Organization, probiotics are "living organisms, which when administrated in adequate amounts, confer a health benefit to the host" (Parkes G. C. *et al.*, 2009). Several studies investigating the use of probiotic to treat and/or prevent CDI have led to inconclusive and often contradictory results. Common probiotics investigated often contain microorganisms such as *Lactobacillus* species and yeast like *Saccharmoyces boulardii*. In a recent study, neonatal piglets were intragastrically administered with a single dose of lactobacillus and non-toxigenic *C. difficile* isolate respectively, and later challenged with a toxigenic isolate. Results from this study revealed that the use of non-toxigenic *C. difficile* as a probiotic reduced the severity of histologic lesions associated with CDI and the amount-of toxins detected in neonatal piglets. Use of *Lactobacillus* species did not yield any form of protection against challenge, and animals were statistically undistinguishable from piglets receiving toxigenic strains of *C. difficile* alone *(Arruda et al. 2014, submitted)*.

Antibiotic treatment is the most common treatment utilized for CDI in humans. Vancomycin and metronidazole are the drugs of choice. Studies evaluating the antimicrobial susceptibility of *C. difficile* isolates originating from confirmed CDI piglet cases are lacking.

CHAPTER 2. EFFECT OF AGE, DOSE AND ANTIBIOTIC THERAPY ON THE DEVELOPMENT OF *CLOSTRIDIUM DIFFICILE* INFECTION IN NEONATAL PIGLETS

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Abstract

Piglet diarrhea is associated with increased pre-weaning mortality, poor growth rates, and variation in weight at weaning. *Clostridium difficile* is a known cause of enteric disease in neonatal piglets, yet risk factors associated with *C. difficile* infection in piglets are unknown. The objectives of this study were (1) to evaluate the consistency and severity of lesions in piglets challenged with *C. difficile* at different bacterial doses (DOSAGE experiment), (2) evaluate the use of antibiotics as a contributing risk factor in 1-day-old piglets (ANTIMICROBIAL experiment), and (3) to provide a clinical and histological evaluation of *C. difficile* infection in 10-day-old piglets (AGE experiment). One hundred and eleven conventional neonatal pigs were snatch farrowed and divided into experimental groups addressing the objectives. In the DOSAGE experiment, 40 1-day-old piglets were sham inoculated or challenged with varying amounts of *C. difficile* heat shocked spores and euthanized 72 h post infection. Results indicate a clear trend for disease development as bacterial numbers increase. In the ANTIMICROBIAL experiment, 39 1-day-old piglets were challenged and then treated with one of four different antibiotics after 16 h. No significant difference in disease development was found. Thirty-three 10-day-old piglets were given varying doses of *C. difficile* in the AGE experiment. Disease and lesions were reproduced in

10 day-old piglets. Combined results indicate that *C. difficile* dosage appears to be an important factor that influences the appearance and severity of lesions, 10 day-old pigs can develop disease associated with *Clostridium difficile*, and antibiotic administration following inoculation may not be a major contributor for disease in neonatal piglets.

Introduction

Clostridium difficile is a Gram positive, anaerobic, spore-forming bacterium first described as part of the neonatal intestinal flora in 1935 [1]. Subsequently, in 1978, *Clostridium difficile* was linked to human colitis [2], and is now a significant cause of antibiotic-associated diarrhea in several countries worldwide [3-8]. *Clostridium difficile* infection (CDI) in humans is characterized by mild to severe diarrhea, pseudomembranous colitis, and, in the most severe cases, by paralytic ileus, toxic megacolon, bowel perforation, peritonitis, and death. CDI has also been described in several non-human species including pigs, horses, primates, rabbits, rats, domestic dogs and domestic cats [1,9-12]; disease is typically life-threatening only in horses.

The incidence of CDI has been steadily increasing in veterinary medicine. The majority of cases are associated with disequilibrium of commensal intestinal flora. Neonates, as well as animals treated with select antimicrobials, are most commonly affected [13,14], and the hypothesis of causation is that antimicrobials eliminate susceptible microflora within the intestine, thereby allowing strains of *C. difficile* to establish in empty niches and overgrow due to lack of competition.

CDI in piglets is associated with large bowel inflammation, with the potential for pseudomembrane formation. Gnotobiotic pigs have been described as also having systemic disease resulting in ascites, pleural effusion, hepatic abscess, renal dysfunction, and acute

respiratory distress [8]. The mechanism by which the microorganism causes systemic disease is not completely understood. Toxin A (TcdA), toxin B (TcdB), and binary toxin (CDT) are known products of many strains of *C. difficile*. TcdA and TcdB are large polypeptides, which are believed to be essential virulence factors associated with disease development [1,15]. TcdA is known for enterotoxicity while TcdB is a potent cytotoxin and enterotoxin *in vivo* [5]. There are reports of failed disease associated with TcdB *in vivo* unless prior cellular damage by TcdA has occurred [5,16]. The significance and accuracy of these observations is in question, especially due to the occurrence of human infections with $TcdA^{-}TcdB^{+}$ strains. A mouse study demonstrated that TcdB is several times more toxic than TcdA and *C. difficile* isolates lacking TcdA are still capable of causing severe disease [17]. These results contradict the theory that TcdA action on tissues is an essential prerequisite to cell damage by TcdB. This is further supported by a more recent study using TcdA or TcdB gene knock out *C. difficile* strains. Reported results from this study indicate both are able to cause cellular alterations *in vitro* and *in vivo* disease in hamsters, highlighting the fact that both toxins are independently capable of producing disease [18].

Clostridium difficile intestinal colonization occurs within the first hours of life in the neonatal pig, and nearly one hundred percent of piglets in some herds are colonized within 48 h of birth [11]. In contrast to colonization, CDI does not affect all piglets within a litter, but can manifest as mild to severe diarrhea in 1-7 day-old piglets [11,19]. Neonatal piglets are highly susceptible to colonization, as the intestinal microflora is not fully established. This establishes piglets as a good model for human studies but also presents a serious problem in the swine industry.

Many risk factors are thought to contribute to CDI, including administration of antimicrobials, dose, associated toxin profile, and animal age. However, there is a lack of knowledge regarding these aforementioned risk factors in swine. The objectives of this study were (1) to evaluate the consistency and severity of lesions in piglets challenged with different bacterial doses, (2) to evaluate the use of antimicrobials as a contributing risk factor in the development of disease, and (3) to provide a clinical and histological evaluation of *C. difficile* infection in 10-day-old piglets.

Material and Methods

Animals

One hundred and eleven conventional neonatal pigs were procured from a 2,500 head sow farm located in central Iowa. Procedures involving sow preparation, piglet collection and care were as previously described [20]. Once piglets have been collected and properly processed, colostrum collection and administration was performed as described [20]. Pigs were then transported to a BSL-2 animal facility at Iowa State University. Sera from all procured neonatal pigs were negative for porcine reproductive and respiratory virus (PRRS) nucleic acid by PCR. Serum was analyzed for PRRSV nucleic acids using a licensed real time PCR assay (Applied Biosystems, CA, USA).

Housing

Piglets were housed in one of six identical raised plastic tubs partitioned into eight individual pens (approximately 0.7 x 0.7 m) with clear solid plastic dividing walls. Piglets housing and daily care were as described previously [20] with slight modifications. Prior to piglet arrival, rooms and plastic tubs were cleaned with total removal of organic material and disinfected with 2% potassium peroxymonosulfate (Virkon® S, DuPont; Wilmington, DE)

for a 4 h period to effectively eliminate environmental vegetative cells and spores. All challenged pigs were housed in the same room and airspace. Negative control piglets were housed separately.

Experimental Design

Three separate experiments were completed. Table 1 summarizes the design for all three experiments. Each experiment was a specific objective to be investigated: bacterial dosage was evaluated in experiment 1 (DOSAGE), antimicrobial usage and the development of disease in experiment 2 (ANTIMICROBIAL), and the effect of piglet age in experiment 3 (AGE). In each experiment, pigs were randomly allocated into four (DOSAGE and AGE) or five (ANTIMICROBIAL) groups using several random number iterations in Microsoft Excel®. The experimental protocol was approved by the Iowa State University Institutional Animal Care and Use Committee (#9-10-7014-S). In the DOSAGE experiment, three different quantities of heat shocked *C. difficile* spores were inoculated. Group 1 received sham material and groups 2, 3, and 4 received $2x10^3$, $2x10^6$, $2x10^9$ heat-shocked *Clostridium difficile* spores, respectively (Table 1). For the ANTIMICROBIAL experiment, all groups were challenged with $2x10^6$ heat-shocked *Clostridium difficile* spores. Sixteen hours following *C. difficile* challenge, groups 6, 7, 8, and 9 were each administered a different antibiotic (Table 1). The AGE experiment utilized the same protocol for piglet collection. Piglets were kept for 10 days and then challenged with $2x10^3$, $2x10^6$, or $2x10^9$ *C. difficile* spores (Table 1). Piglets were euthanized 72 h after challenge in all experiments.

Inoculum

C. difficile isolate ISU-15454-1, was used for all experiments. This isolate originated from a field case of neonatal diarrhea in 3-6 day-old piglets. High levels of toxin A and/or B

(4+) were detected by ELISA (C. DIFFICILE TOX A/B II™, Blacksburg, VA) from the clinically affected piglets. Isolate 15454-1 is ribotype 078, toxinotype V, and contains both toxin A and toxin B gene sequences [21]. The isolate was stored at -80°C until culture preparation. Procedures involving *C. difficile* isolation, growth harvesting, and titration of spores was accomplished as previously described [20].

Inoculation

All piglets were inoculated intragastrically using an eight-gauge rubber French catheter as an oral-gastric tube (Sovereign ™, Tyco/Healthcare, Mansfield, MA). Inoculation occurred approximately four h after birth in the DOSAGE and ANTIMICROBIAL experiments, and 10 days for the AGE experiment. The negative control groups in the DOSAGE and AGE experiments were given 1.25 ml of sterile nanopure water, and then flushed with 20 ml of milk replacement. For all inoculated groups in the DOSAGE, ANTIMICORBIAL, and AGE experiments, 1.25 ml of challenge preparation containing heat-shocked *Clostridium difficile* spores [20] was given followed by 20 ml of milk replacement.

Antimicrobials

Sixteen hours post-inoculation, select groups in the ANTIMICROBIAL experiment (Table 1) were injected intra-muscularly with one of four antibiotics commonly used in the swine industry. The subsequent antibiotics were used: Lincomycin (Lincocin®, Pfizer Animal Health, New York, NY), Ceftiour (Excede®, Pfizer Animal Health, New York, NY), Tylosin (Tylan®, Elanco Animal Health, Greenfield, IN) and Tulathromycin (Draxxin®, Pfizer Animal Health, New York, NY). All antibiotics were administered per labeled directions.

Necropsy

Piglets from all experiments were monitored for 72 h post-challenge and then euthanized by an intravenous overdose of pentobarbital. Gross observations at necropsy included 1) body condition, 2) dehydration status, 3) perineal fecal staining, 5) consistency of colonic contents, 6) mesocolonic edema, and the presence of 7) visible gross colonic luminal necrosis and were scored independently in a blinded fashion as previously described [20,22]. Necropsies, clinical sign scores and gross lesion scores were completed by the same two individuals for all experiments (PHEA and DMM).

Sample collection

Immediately prior to inoculation rectal swabs were taken from all pigs. At necropsy, fresh and formalin-fixed tissues were collected with flamed instruments soaked in 70% alcohol and included ileum, jejunum, descending colon, cecum, and a cross section of spiral colon containing 4-5 loops. Colonic and cecal contents were collected in a sterile plastic cup. A luminal swab (Dacron® Fiber Tipped, Fisher brand®, Leicestershire, UK) of the ileum was also taken.

Toxin detection and culture

Rectal swabs collected prior to inoculation and pooled colon and cecum contents retrieved at necropsy were assayed for *C. difficile* toxins with a commercially available toxin ELISA kit (C. DIFFICILE TOX A/B II™, Blacksburg, VA) used according to manufacturer instructions and analyzed on a microplate reader (IDEXX Corp, Molecular Device, Lake Forest, IL) to semi-quantitatively grade the amount of toxin from 0 (no toxin detection) to 4+ (marked toxin detection) as indicated by the manufacturer. Toxin ELISA was performed within 4 h following sample collection.

Pre-inoculation rectal swabs, and post-euthanasia pooled colonic and cecal contents were cultured on *Clostridium difficile* selective agar, both directly and following a 30 minute room temperature incubation in 0.5 ml absolute ethanol [20]. All plates were incubated at 37°C for 48 h in an anaerobic chamber. *C. difficile* growth following incubation was semi-quantitatively scored in a blinded manner by a veterinary microbiologist as follows: 0 = no growth, 1 = few colonies, 2 = low numbers of colonies, 3 = moderate growth and 4= high growth [20]. Luminal swabs collected at necropsy from the small intestine were examined by routine aerobic and anaerobic culture methods for *Salmonella* spp, *Escherichia coli*, and *Clostridium perfringens*. Genotyping for *E. coli*, and *C. perfringens* were performed [23,24] to determine surface antigen and associated toxin genes.

Histopathology

Tissue sections were collected in 10% neutral buffered formalin and allowed to fix for 24h. Tissues were then placed in 70% ethanol until routine tissue sectioning followed by paraffin embedding and staining with hematoxylin and eosin. Large intestinal sections were assessed for goblet cell loss, neutrophilic aggregates within the lamina propria, and mucosal epithelial defects as previously described [20].

Scoring

Three categories of scores were compared: 1) clinical signs, 2) gross lesions, and 3) microscopic lesions. The scoring system was as previously described [20].Clinical sign scores were created by summing scores for body condition, hydration status, and perineum staining which ranged from 0 (normal) to three (severe) for each category. Gross lesion scores were created by summing scores for necrotizing lesions, mesocolonic edema, culture result for *C. difficile*, and toxin amount. Microscopic lesion score was the sum of scores for

all histopathology categories. Briefly, large intestinal segments were microscopically assessed for goblet cell loss, the quantity of infiltrating neutrophils within the lamina propria, mucosal alterations, and mesenteric inflammation. Each microscopic category was scored 0 (normal) to three (severe) depending on severity as previously described [20].

Statistical methods

Scores for clinical signs and gross and microscopic lesions were analyzed by a non-parametric test. Wilcoxon/Kruskal test was used to determine if differences existed between comparisons of control groups to isolate groups, and isolate groups to each other. JMP 9 (JMP®, Cary, NC) statistical software was used to perform analyses.

Results

Clinical signs

Clinical scores were independently scored for all pigs within their respective groups and experiments. Results are summarized in Table 2. Statistical evaluation of clinical signs scores from DOSAGE, ANTIMICROBIAL, and AGE experiments revealed no statistical difference ($p>0.05$) among their respective groups. The majority of pigs at necropsy in the DOSAGE experiment presented with normal body condition as well as hydration status with exception of one animal challenged at $2x10^6$, which presented with moderate levels of dehydration. Forty percent (4/10) of piglets challenged at $2 x10^3$ *C. difficile* spores, 50% (5/10) of $2x10^6$, and 80% (8/10) of $2x10^9$ presented with staining of perineum at necropsy. Thirty percent (3/10) of control pigs also had mild staining.

In the ANTIMICROBIAL experiment, most piglets were mildly to severely dehydrated (37/39, 94.9%) at 72 h post inoculation and had moderate to marked fecal

staining of the perineum (38/39, 97.4%). All but one or two piglets in each group (33/39, 84.6%) were noted to be thin or emaciated.

Piglets from all groups in the AGE experiment were of normal body condition, hydration status, and had no perineal fecal staining 72 h post inoculation with few exceptions. One (1/7, 14.3%) piglet in group 10 was thin and two (2/9, 22.2%) piglets in group 13 had fecal staining of the perineum.

Gross lesions

Statistical differences were not detected between groups of the DOSAGE, ANTIMICROBIAL, and AGE experiments. Grossly visible mucosal necrosis was not seen within the cecum or spiral colon of individual piglets in all experiments. Gross lesions by experiment are summarized in Table 3. The gross lesions score for the DOSAGE experiment were not statistically different between groups. However, the score of group 1 was numerically lower than groups 2, 3, and 4. No mesocolonic edema was reported in group 1. Mesocolonic edema presented in 30% (3/10) of group 2 piglets and 40% (4/10) of piglets in groups 3 and 4.

Within the ANTIMICROBIAL experiment, mesocolonic edema was reported at different frequencies among all groups. Edema was observed in one (1/8, 12.5%) pig each from groups 5, 6, and 8. Two (2/8, 25%) piglets in group 7 and four (4/7, 57.1%) from group 9 had mesocolonic edema 72 h post inoculation.

Five (5/7, 71.4%) piglets in group 10 of the AGE experiment developed mild mesocolonic edema. In group 11, four (4/8, 50%) piglets had edema. Three (3/9, 33.3%) and five (5/9, 55.6%) piglets had mesocolonic edema at necropsy in groups 12 and 13, respectively.

Microscopic lesions

Classical microscopic lesions with high numbers of neutrophils infiltrating the lamina propria, loss of goblet cells and the presence of single to multiple sites of erosions and/or ulcerations were observed in sections of colon and cecum from *C. difficile* challenged piglets in the DOSAGE, ANTIMICROBIAL, and AGE experiments. Observed microscopic lesions for all experiments are detailed in Table 4. Significant differences between groups were not observed in the DOSAGE, ANTIMICROBIAL, and AGE experiments.

For the DOSAGE study, overall microscopic lesions scores were not statistically different between groups (p=0.2), however, there is a clear numeric trend between dose of *C. difficile* and associated microscopic lesions (Figure 1). Lesion severity was elevated in animals challenged at higher doses when compared to lower doses and negative piglets. Only rare aggregates of neutrophils were seen within the group 1 piglets. No goblet cell loss or mucosal alterations were apparent. Small intestinal bacterial adherence suggestive of *Escherichia coli* was not observed in any pig from all experiments.

Toxin ELISA

Prior to *C. difficile* inoculation, rectal swabs from all pigs in the DOSAGE ANTIMICROBIAL, and AGE experiments were negative for both toxins A and/or B. Toxin ELISA at termination of experiments is summarized in Table 5.

All the group 1 piglets in the DOSAGE experiment remained toxin negative. Fifty percent (5/10) of group 2 piglets were toxin positive at necropsy. Moderate to high levels of toxin were detected in four of the five (80%) piglets. Similarly, 50% (5/10) of group 3 piglets were toxin positive 72 h after inoculation. Toxin quantity varied from in these five piglets.

Alternatively, 90% (9/10) of group 4 piglets were toxin positive 72 h following inoculation. All positive pigs in group 4 had moderate to high levels, (3 or 4+), of toxin present.

Toxin levels were not detected in any group 6 (0/8) piglet from the ANTIMICROBIAL experiment. Low to moderate levels of toxin were detected in three (3/8, 37.5%) group 7 piglets. Two (2/8, 25%) group 8 and one (1/7, 14.3%) group 9 piglet had low amounts of *C. difficile* toxin present at 72 h post inoculation. Within the positive control piglets, group 5, one (1/8, 12.5%) piglet was toxin positive. However, contrary to other groups, toxin was detected at high levels (Table 5).

In the AGE experiment, no (0/7, 0%) group 10 piglets were positive for *C. difficile* toxin at necropsy. Toxin was detected in three (3/8, 37.5%) group 11 piglets. Two (2/9, 22.2%) piglets from group 12 and 13 had detectable toxin.

Bacterial Culture

Clostridium difficile culture results for the DOSAGE, ANTIMICROBIAL, and AGE experiments are summarized in Table 6. No *Salmonella* spp was isolated from any pig. Twelve *C. perfringens* isolates were randomly selected for PCR, and all were determined to be type A. Genes associated with Beta 2 toxin were found in three (3/12, 25%) isolates; all others were negative. Twelve hemolytic and non-hemolytic *E. coli* isolates were randomly selected and were all negative for pilus antigen and associated toxin genes.

Discussion

Several studies have reported that neonatal piglets are susceptible to *C. difficile* toxin which results in yellow pasty diarrhea [8,25,26]. Pre-weaning mortality, poor growth rates and variation in piglet weight at weaning are common problems associated with CDI [27]. Although the awareness of this disease has increased in swine production over the last

decade, more research is needed to better understand basic principles such as prevention, risk factors, epidemiology and treatment. The treatment of affected animals is occasionally complicated by the fact that this bacterium is resistant to different classes of antibiotics [28]. A better understanding of the risk factors or triggers is very important in order to effectively block or minimize the occurrence of the disease because there is a lack of commercial preventive products for CDI.

Microscopic results of the DOSAGE experiment suggest amplified CDI prevalence and severity with increasing exposure dose. It was believed that *C. difficile* shed by sows in farrowing crates was the main route of exposure for neonatal pigs. Although, a recent study reported that only about 25% of sows tested were actively shedding the organisms during lactation [12]. Studies investigating the source of *C. difficile* have concluded that neonatal pigs, ambient air, and the environment are the major sources of piglet exposure [11,29]. *C. difficile* spores are highly resistant to physical and chemical agents such as farm cleaning procedures and most common disinfectants [30,31] and can survive for many months in the environment [32]. Therefore, we hypothesize that decreasing the exposure dose to piglets in the first day of life and allowing colonization of other microbiome species could have a significant impact in disease occurrence as well as disease severity.

Clostridium difficile is the major cause of antibiotic-associated diarrhea in humans [3-8] and the same phenomenon is observed in veterinary medicine, where the majority of cases are associated with disequilibrium of common intestinal microflora, due to antibiotic treatment or young age of animals [33]. A recent study done in mice reported that the use of antimicrobials is a critical element in order to cause disease and histopathologic lesions [34]. Piglet CDI is thought to be a spontaneous disease with no real antimicrobial interaction

[22,35,36], but confirmation is lacking. In the current study, microscopic lesions associated with CDI were observed in piglets enrolled in the ANTIBIOTIC experiment; however, no statistical difference was demonstrated between groups. This finding suggests that antibiotic treatment, as applied, does not play an important role in the development of and/or in the severity of CDI lesions in piglets.

One day-old piglets do not have an established intestinal microflora, and therefore antibiotics would not significantly alter the microflora. This is a potential reason for antibiotic therapy not influencing disease severity in the current study. Another debate is that the isolate used in this study was susceptible to used antimicrobials reducing the influence of treatment. A considerable percentage of piglets involved in this experiment were toxin negative and culture negative at the end of the experiment (Table 5). The antibiotic resistance profile of *C. difficile* strains associated with human disease is constantly investigated [37,38]; however, studies investigating the resistance profile of piglet *C. difficile* isolates are lacking. A different, susceptible *C. difficile* isolates could potentially enhance disease severity. Piglets treated with antibiotics did, however, have more clinical diarrhea than expected, but a reasonable conclusion could not be drawn for this finding. Nonetheless, we believe that a better understanding of the role played by antimicrobials in the dynamic of microorganism colonization, succession, and competition in the first day of life could contribute to the knowledge of neonatal intestinal diseases and the establishment of a healthy microflora.

There is consensus in the literature that a large majority of cases of CDI in piglets occur within the first five days of life [25]. In the AGE experiment, CDI was induced in 10-day old piglets, indicating susceptibility. The results from this experiment suggest that the challenge dose does not correlate with development and severity of disease in older piglets as

it does in one day-old piglets. However, these data should be interpreted with caution since piglets were kept in a controlled environment until they reached 10 days of age. This perhaps limited, and potentially altered, the normal colonization of gut flora which would typically have occurred in piglets raised in the farm environment. Another factor that differentiates experimental piglets from piglets raised on farm is milk and its rich source of antibodies. These antibodies are responsible for mucosal protection and possibly play a role in bacterial colonization and growth. Unexpectedly, the total microscopic score was relatively high in the control group. A single piglet in this group had significant microscopic lesions, accounting for more than half of the total score of the group. However, this was toxin negative and was *C. difficile* culture negative at the end of the study. Therefore we suspect that microscopic lesions are likely not due to *C. difficile* infection although possible cause of lesions could not be determined at this point.

TcdA and TcdB are believed to be the main factors in disease development [15,39]. Toxin ELISA test was performed on piglet feces with the objective of quantifying the toxin levels. We were not able to demonstrate a correlation between ELISA results and microscopic lesions, and these results were consistent across the three different experiments. The effects of *C. difficile* toxins on enterocytes has been well described[1,5], therefore we hypothesize that this failure of correlation might be due to the lack of sensitivity of the ELISA used in this study [39]. Furthermore, ELISAs commonly used in veterinary medicine were primarily designed to human cases; therefore, any difference which might exist between piglet and human cases could potential influence the results.

Recently, several articles have investigated the use of ELISA as a diagnostic tool for CDI in humans. The consensus is that the ELISA should not be used as a sole diagnostic tool

due to the wide range in sensitivity and specificity as well as poor positive predictive value [40,41]. The ELISA test used in the study identifies the total amount of toxin A and B within sample; the test is not capable to differentiating which particular toxin is present within the sample. Therefore, it is not possible to extrapolate the role a particular toxin played on disease development or the prevalence of toxins within studied animals. As a way to improve CDI diagnosis, hospitals are introducing multiple testing algorithms which use ELISA associated with other tests such as PCR (targeting genes responsible for toxin production) and the glutamate dehydrogenase assay, with the objective of increasing sensitivity and specificity.

In swine medicine, ELISAs are still the most widely used diagnostic tools and are frequently the only diagnostic tool used. The lack of correlation in our results, as well as the poor sensitivity performance in human medicine, we believe that new CDI diagnostics, such as immunohistochemistry and molecular assays, are needed in veterinary diagnostic laboratories. Based on the results from these studies, clinical evaluation of piglets is not a good measure to evaluate disease development or severity; microscopic lesions appear to be the most appropriate method to evaluate such parameters.

The *C. difficile* model used for these experiments is predictable and repeatable [20]; however, there are multiple limitations. Piglet procurement was from a commercial sow farm, and although multiple precautions were taken, all piglets were potentially infected with endemic microorganisms [20]. This was important for these experiments as the dynamic of microorganism succession and competition is believed to be the key factor for disease development. The farm had no previous history of CDI in piglets, but few control piglets were found to be colonized with *C. difficile*, which correspond to the current literature that

piglets start to become colonized within a few hours post-farrowing, and within 48 h of life 100% of farm-raised piglets are colonized [11]. All *C. difficile* isolates from control pigs were toxin negative throughout the study period. Cesarean-surgery piglet derivement would limit this potential hazard in future repetitions of the model. Additionally, the large normal variation in measured variables, the short study period and the small numbers of piglets per group might have contributed to the lack of statistically significant findings of this study.

In conclusion, the results demonstrate that our isolate can cause microscopic lesions and the challenge dose appears to be an important factor that influences the development and severity of lesions. In this study, antimicrobial administration appeared to not influence the appearance and severity of lesions. It was also demonstrated that 10 day-old-piglets are susceptible to the developmental of CDI under these study circumstances. More studies are needed to better understand risk factors, epidemiology, and the contribution of antimicrobial treatment to the prevalence and severity of *C. difficile* disease in piglets.

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Tables and Figures

Table1. Experimental design.

Experiment	Groups	*n*	Age	Inoculation & dose‡	Treatment*
DOSAGE	1	10	1 day	None (control)	N/A
	2	10		2 x10^3 *C. difficile* spores	
	3	10		2 x 10^6 *C. difficile* spores	
	4	10		2 x 10^9 *C. difficile* spores	
ANTIMICROBIAL	5	8	1 day	2 x 10^6 *C. difficile* spores	None
	6	8		2 x 10^6 *C. difficile* spores	Lincomycin
	7	8		2 x 10^6 *C. difficile* spores	Ceftiofur
	8	8		2 x 10^6 *C. difficile* spores	Tylosin
	9	7		2 x 10^6 *C. difficile* spores	Tulathromycin
AGE	10	7	10 days	None (control)	N/A
	11	8		2 x10^3 *C. difficile* spores	
	12	9		2 x 10^6 *C. difficile* spores	
	13	9		2 x 10^9 *C. difficile* spores	

‡ Heat-shocked *Clostridium difficile* spores

* Antibiotic doses were administered per label based on weight and given as directed intramuscularly 16 h post inoculation

Table 2. Clinical signs by experiment and group. Clinical signs scores included body condition, hydration status, and perineal fecal staining at 72 h post inoculation with sham or heat shocked *Clostridium difficile* spores.

Clinical signs			Body Condition		Hydration Status		Perineum Staining		Sum of scores
Experiment	Group	*n*	Range	Mean (+/- SE)	Range	Mean (+/- SE)	Range	Mean (+/- SE)	Mean
DOSAGE	1	10	0-1	0.2 (0.13)	0-1	0.3(0.15)	0-1	0.3(0.15)	1.8
	2	10	0	0 (0)	0-1	0.3(0.15)	0-2	0.7(0.3)	1.0
	3	10	0-1	0.2 (0.13)	0-2	0.6(0.22)	0-2	0.7(0.26)	1.5
	4	10	0-1	0.2 (0.13)	0-1	0.4(0.16)	0-2	1(0.21)	1.6
ANTIMICROBIAL	5	8	0-2	1.1(0.22)	0-3	1.7(0.36)	1-3	2.2(0.36)	5.1
	6	8	0-2	1.5(0.32)	0-3	1.8(0.44)	1-3	2.6(0.26)	6
	7	8	0-2	1.5(0.26)	1-3	2(0.37)	0-3	2.1(0.39)	5.6
	8	7	0-2	1.14(0.26)	1-3	2(0.3)	1-3	2.5(0.29)	5.8
	9	8	0-2	1.2(0.25)	1-3	2.1(0.64)	2-3	2.6(0.18)	6
AGE	10	7	0	0(0)	0(0)	0	0	0(0)	0
	11	8	0	0(0)	0	0(0)	0	0(0)	0
	12	9	0	0(0)	0	0(0)	0	0(0)	0
	13	9	0-1	0.1(0.1)	0-1	0.2(0.14)	0-4	0.2(0.29)	0.8

Table 3. Gross lesion scores, with culture and toxin results, by experiment and group on pigs challenged with heat shocked spores *C. difficile*

Gross Lesions			Mesocolonic Edema		Culture		Toxin		Sum of scores
Experiment	Group	*n*	Range	Mean (+/- SE)	Range	Mean (+/- SE)	Range	Mean (+/- SE)	Mean
DOSAGE	1	10	0	0(0)	0-2	1.1(0.27)	0	0(0)	1.1
	2	10	0-3	0.5(0.3)	0-3	1.3(0.33)	0-4	1.7(0.59)	3.5
	3	10	0-3	0.8(0.35)	0-3	0.4(0.3)	0-4	1.3(0.49)	2.5
	4	10	0-3	1(0.44)	0-3	2.4(0.3)	0-4	3(0.36)	6.4
ANTIMICROBIAL	5	8	0-3	0.37(0.37)	0-2	1.2(0.25)	0-4	0.5(0.5)	2.1
	6	8	0-2	0.25(0.25)	0-1	0.12(0.12)	0	0(0)	0.3
	7	8	0-3	0.5(0.37)	0-2	1(0.32)	0-2	0.6(0.32)	2.1
	8	7	0-1	0.14(0.14)	0-1	0.5(0.2)	0-1	0.28(0.18)	0.9
	9	8	0-1	0.5(0.18)	0-2	0.3(0.26)	0-1	0.12(0.12)	1
AGE	10	7	0-1	0.71(0.18)	0-1	0.5(0.2)	0	0(0)	1.3
	11	8	0-3	0.87(0.39)	0-3	1.8(0.39)	0-2	0.5(0.26)	3.3
	12	9	0-2	0.44(0.24)	0-4	2.3(0.37)	0-3	0.4(0.33)	3.2
	13	9	0-3	0.77(0.32)	0-3	2(0.33)	0-3	0.5(0.37)	3.3

Table 4. Microscopic lesions 72 h post inoculation in large intestine of piglets sham challenged or challenged with heat shocked *Clostridium difficile* spores by experiment and group.

Microscopic Lesions											
			Goblet cell loss		Neutrophilic inflammation		Mucosal defects*		Mesenteritis		Sum of scores
Experiment	Group	*n*	Range	Mean (+/- SE)	Range	Mean (+/- SE)	Range	Mean (+/- SE)	Range	Mean (+/- SE)	Mean
DOSAGE	1	10	0	0(0)	0-2	0.7(0.26)	0	0(0)	0	0(0)	0.8
	2	10	0-3	0.8(0.41)	0-3	0.7(0.33)	0-3	0.4(0.3)	0-1	0.1((0.1)	2.6
	3	10	0-3	0.9(0.37)	0-3	1.2(0.41)	0-3	0.9(0.4)	0-2	0.4(0.22)	4.7
	4	10	0-3	1.4(0.42)	0-3	1.4(0.42)	0-3	1.2(0.44)	0-2	0.7(0.26)	5.5
ANTIMICROBIAL	5	8	0-3	0.75(0.41)	0-3	0.87(0.39)	0-3	0.5(0.37)	0-1	0.25(0.16)	3.8
	6	8	0-3	0.5(0.37)	0-3	0.62(0.41)	0-2	0.25(0.25)	0-1	0.12(0.12)	1.8
	7	8	0-3	0.37(0.37)	0-3	0.5(0.37)	0-3	0.37(0.37)	0-1	0.12(0.12)	3
	8	7	0-2	0.28(0.28)	0-2	0.57(0.29)	0-1	0.14(0.14)	0-1	0.14(0.14)	2.1
	9	8	0-1	0.25(0.16)	0-2	0.5(0.26)	0-1	0.12(0.12)	0	0(0)	1.3
AGE	10	7	0-2	0.71(0.36)	0-3	1.14(0.53)	0-3	0.71(0.47)	0-1	0.14(0.14)	4.1
	11	8	0-2	0.25(0.25)	0-3	0.62(0.37)	0-1	0.12(0.12)	0-3	0.5(0.37)	2.8
	12	9	0-1	0.11(0.11)	0-1	0.33(0.16)	0	0(0)	0-3	0.77(0.36)	1.2
	13	9	0-3	0.88(0.45)	0-3	1.11(0.48)	0-3	0.88(0.45)	0-3	1(0.44)	4.7

*Mucosal defects include erosions and/or ulceration

Table 5. *Clostridium difficile* culture and toxin ELISA results by experiment from pigs challenged with heat shocked *Clostridium difficile* spores (culture and toxin ELISA were performed on pooled large intestinal contents and colonic mucosal scrapings).

Experiment	Group	*C. difficile* culture*	Toxin ELISA†	Treatment¶
DOSAGE	1	2/10	0/10	.
	2	7/10	5/10	.
	3	2/10	5/10	.
	4	9/10	9/10	.
ANTIMICROBIAL	5	6/8	7/8	.
	6	0/8	1/8	Lincomycin
	7	3/8	5/8	Ceftiofur
	8	0/7	4/7	Tylocin
	9	1/8	2/8	Tulathromycin
AGE	10	4/7	0/7	.
	11	7/8	1/8	.
	12	8/9	3/8	.
	13	7/9	2/9	.

* Number of samples culture positive for *C. difficile* per total number of piglets within each group.

† Number of samples toxin positive for *C. difficile* per total number of piglets within each group.

¶ Only piglets enrolled in the ANTIMICROBIAL experiment were treated; 16 h post inoculation with *C. difficile*.

Table 6. Small intestine culture results by experiment from pigs challenged with heat shocked *Clostridium difficile* spores (luminal swabs from the ileum were cultured at necropsy).

Experiment	Group	Hemolytic *E. coli**	Mucoid/ Smooth *E. coli* ‡	*C. perf* †	Treatment¶
DOSAGE	1	0/10	0/10	2/10	.
	2	0/10	3/10	0/10	.
	3	2/10	1/10	0/10	.
	4	1/10	2/10	0/10	.
ANTIMICROBIAL	5	2/8	1/8	1/8	.
	6	7/8	1/8	1/8	Lincomycin
	7	5/8	1/8	1/8	Ceftiofur
	8	1/7	1/7	1/7	Tylocin
	9	1/8	1/8	0/8	Tulathromycin
AGE	10	3/7	1/7	0/7	.
	11	5/8	4/8	0/8	.
	12	1/8	1/8	0/8	.
	13	4/9	0/9	0/9	.

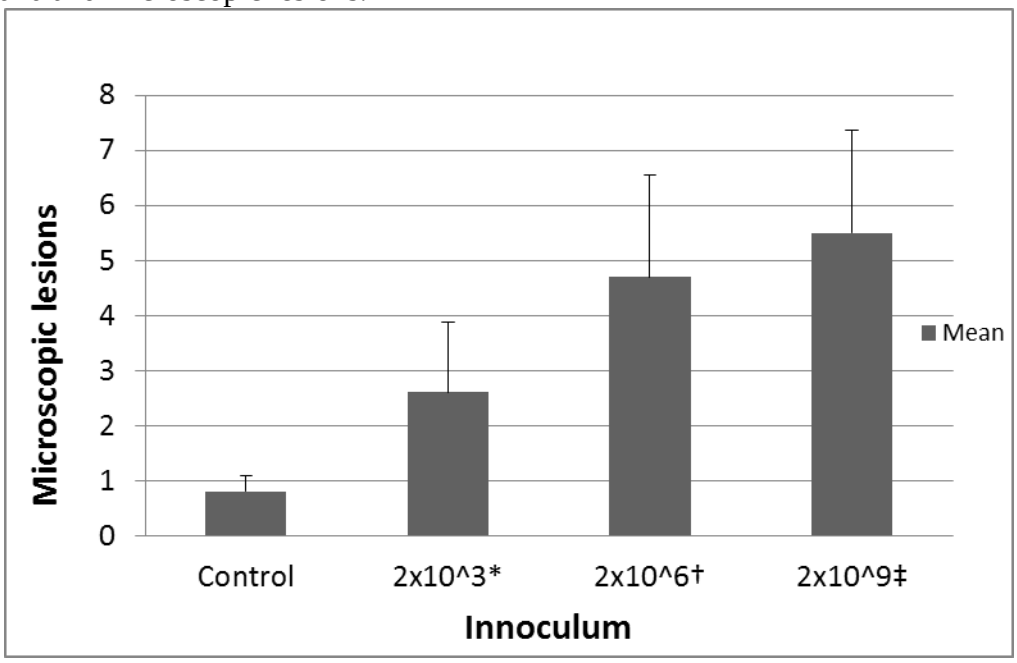
* Number of samples culture positive for hemolytic *Escherichia coli* per total number of piglets within each group.

† Number of samples culture positive for *Clostridium perfringens* per total number of piglets within each group.

‡ Number of samples culture positive for smooth or mucoid *Escherichia coli* per total number of piglets within each group.

¶ Only piglets enrolled in the ANTIMICROBIAL experiment were treated; 16 h post inoculation with *Clostridium difficile*.

Figure 1. Mean microscopic lesions by group for the DOSAGE experiment. There were no significant differences between groups; however, there is a clear trend with increased dose of *C. difficile* and microscopic lesions.



* $2x10^3$ heat-shocked *Clostridium difficile* spores
† $2x10^6$ heat-shocked *Clostridium difficile* spores
‡ $2x10^9$ heat-shocked *Clostridium difficile* spores
Mean: sum of the different Histopathologic categories

CHAPTER 3. BACTERIAL PROBIOTICS AS AN AID IN THE CONTROL OF *CLOSTRIDIUM DIFFICILE* ASSOCIATED DISEASE IN NEONATAL PIGS

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Abstract

Clostridium difficile infection (CDI) in piglets is associated with large bowel inflammation, with the potential for pseudomembrane formation, and can lead to production losses or increased mortality. Although CDI is a common disease in the swine industry, there is a general lack of prevention strategies. The objectives of this study were to evaluate: (1) the usage of Lactobacillus sp. and (2) non-toxigenic *C. difficile* (NTCD) prevention alternatives in the development of CDI in piglets. One hundred and fifty five cesarean derived piglets were randomly assigned to six different groups as follows: GROUP 1 negative control (n= 10), GROUP 2 NTCD only (n= 13) , GROUP 3 Lactobacillus sp. only (n= 14), GROUP 4 positive control (challenged with toxigenic C. difficile strain) (n= 35), GROUP 5 NTCD and challenged with the toxigenic *C. difficile* strain (n= 34), GROUP 6 Lactobacillus sp. and challenged with the toxigenic *C. difficile* strain (n= 44). Assigned groups according to experimental design received a single dose of selected preventative at 4 hours-of-life and were challenged with toxigenic *C. difficile* sixteen hours later. All piglets were euthanized at 72 hours post infection. Results showed a benefit of the usage of NTCD to prevent CDI in piglets, the prevalence of toxin-positive piglets, mesocolonic edema and histopathologic lesions were reduced when compared to positive control piglets. Usage of Lactobacillus sp. did not reveal clear benefits.

Introduction

Disease associated with *Clostridium difficile*, a Gram positive, anaerobic, spore-forming bacterium, has been described in several species including humans, pigs, horses, non-human primates, rabbits, rats, domestic dogs and cats (Arroyo et al., 2005; Debast et al., 2009; Norman et al., 2009; Hopman et al., 2011; Keessen et al., 2011). The majority of CDI cases are associated with alterations in the gastrointestinal commensal flora, as with antimicrobial therapy, or with undeveloped flora, as in neonatal animals (Rupnik et al., 2009; Lawley et al., 2009). Several studies have shown the role of antimicrobial usage in the development of disease in humans, making CDI the leading cause of antibiotic-associated diarrhea in several countries worldwide (Bartlett, 2002; McDonald et al., 2005; Keel and Songer, 2006; Kelly and LaMont, 2008; Carter et al., 2010; Steele et al., 2010). Discontinued use of antimicrobials (Poutanen and Simor, 2004; Keessen et al., 2011) and probiotic administration are common measures used to control the disease in humans (Parkes et al., 2009; Fitzpatrick, 2013). Alternative techniques to treat disease include the use of fecal transplant (Vaishnavi, 2014) or use of specific bacteria species as probiotics. For instance studies using hamster models have shown that colonization with a non-toxigenic strain of *C. difficile* (NTCD) can prevent CDI (Borriello and Barclay, 1985; Seal et al., 1987; Sambol et al., 2002). Similar results were achieved when NTCD were administered to two human patients suffering from CDI (Seal et al., 1987).

Clostridium difficile is one of the most important enteric pathogens in pigs within the first week-of-life. Songer and others have shown that within affected herds on average two-thirds of the litters are affected, and within the litter the morbidity can be as high as 97-100% (Songer 2004; Anderson and Songer 2008). Mortality rates can vary significantly; however,

mortality as high as 16% have been reported (Anderson and Songer, 2008). Additionally, growth retardation and lower weaning weights in surviving pigs has been reported (Songer, 2004). The newborn piglet is born with a virtually sterile gastrointestinal tract, but colonization by mixed populations of bacteria occurs within hours of birth. Colonizing microbes are mechanically acquired by the piglets via oral contact within the dam's vaginal canal, perineum, teats, exposure to feces, and skin contact (Mackie et al., 1999). Several factors play a role in the dynamic succession of organisms that make up the microflora.

During the piglet's life, several other microbes compete for places in microbial niches in a process of succession that eventually establishes the flora, consisting of well over 500 distinct species of bacteria in the mature gastrointestinal tract (Artis, 2008). Clostridium difficile intestinal colonization occurs within the first hours of life in the neonatal pig, and nearly one hundred percent of piglets in some commercial herds are colonized within 48 h of birth (Hopman et al., 2011). Our research team believes that an intervention during the initial colonization period can prevent CDI in piglets.

Even though CDI is a common disease in the swine industry, there is a lack of sound prevention strategies. Therefore, the objectives of this study were to evaluate the use of (1) Lactobacillus sp. and (2) a non-toxigenic *C. difficile* strain (NTCD) as ingested microorganism (probiotic) alternatives to prevent the development of CDI in piglets.

Materials and Methods

Animals

Pregnant, second to third parity, cross-bred sows from a commercial herd with no history of *C. difficile* disease were purchased and delivered to Iowa State University (ISU) approximately one week prior to expected farrowing. On day 113 of gestation, caesarian

surgeries were performed on sows and the neonatal piglets were manually provided pooled colostrum. All one hundred and fifty-five piglets included in this study were triaged at birth with navels clamped, cut, and sprayed with 5% iodine solution (Durvet, MO, USA). Piglets also received an iron injection at birth. Piglets were kept in a BSL-2 animal facility for the duration of the experiment.

Sera from all neonatal piglets were negative for porcine reproductive and respiratory virus (PRRSV) nucleic acid by PCR. Serum was analyzed for PRRSV nucleic acids using a licensed real time PCR assay (Applied Biosystems, CA, USA).

Housing

Piglets were individually housed in new 18 gallon plastic totes (Rubbermaid®, Port Washington, NY) at a room temperature (29°C); heat lamps were placed above the totes with the objective to increase the microenvironmental temperature of piglets to approximately 35°C. All piglets receiving toxigenic *C. difficile* were housed in the same room and airspace. Negative control, *Lactobacillus* sp. only and NTCD only piglets were housed in separate rooms. Piglet housing and daily care have been described by Arruda et al. (2013). Briefly, piglets were fed milk replacer (Esbilac; Pet-Ag, Hampshire, IL) three times daily (7 am, 12 pm, and 7 pm) by oral-gastric lavage using an 8 French catheter (Sovereign TM, Tyco/Healthcare, Mansfield, MA). At feeding time, piglets were monitored for clinical signs and side effects; piglet behavior, fecal staining and hydration status were evaluated.

Experimental Design

The study design is summarized in Table 1. Briefly, the study contained six separate groups as follows: GROUP 1 negative control (10 piglets), GROUP 2 NTCD only (13 piglets), GROUP 3 Lactobacillus sp. only (14 piglets), GROUP 4 positive control

(challenged with a toxigenic *C. difficile* strain) (35 piglets), GROUP 5 NTCD and challenged with the toxigenic *C. difficile* strain(34 piglets), GROUP 6 Lactobacillus sp. (Probiotic Complex) and challenged with the toxigenic C. difficile strain (44 piglets). Four replicates of the study were performed, summing up to 155 piglets. Two different potentially preventative treatments were utilized: 1) commercially available Lactobacillus sp. and 2) a NTCD strain. In each experiment, pigs were randomly allocated into one of the six groups using several random number iterations in Excel® (Microsoft, Redmond, WA). The experimental protocol was approved by the ISU Institutional Animal Care and Use Committee, protocol number 10-12-7445.

Preventive treatments were administrated intragastrically, according to experimental design, approximately four hours after birth. Piglets from GROUPS 2 and 5 received $2x10^6$ heat-shocked NTCD spores, and piglets from GROUPS 3 and 6 received $2x10^6$ Lactobacillus sp. in a yogurt suspension. Sixteen hours following probiotic administration, groups 4, 5, and 6 were challenged with $2x10^6$ heat-shocked toxigenic *C. difficile* spores (Table 1). Piglets were euthanized 72 h after challenge.

Inoculum

The toxigenic bacterial isolate (*C. difficile* isolate ISU-15454-1) utilized for these experiments was obtained from a field case of piglet diarrhea received at the ISU Diagnostic Laboratory. High levels of toxin (4+) were detected by ELISA (C. DIFFICILE TOX A/B IITM, Blacksburg, VA) from the clinically affected piglet. Isolate 15454-1 is ribotype 078, toxinotype V, and contains both toxin A and toxin B gene sequences (Rupnik et al., 1998). A 1.25 ml of solution containing 2×10^6 heat-shocked spores was intragastrically administered,

followed by 20 ml of milk replacer. The isolate was stored in Chopped meat broth at 3-5∘C; spores from the isolate were prepared and stored at 3-5∘C prior until experimental use. Procedures involving *C. difficile* isolation, growth and harvest of spores have been previously described (Lizer et al., 2013). Furthermore, spore titration and heat shock activation prior to challenge have also been previously described (Lizer et al., 2013).

NTCD

The non-toxigenic strain was kindly provided by Dr. Songer's laboratory. Strain JGS 653 was obtained from a clinically-normal piglet in North Carolina. PCR assays for TcdA and TcdB were negative and toxin production was not detected in 7 day dialysis bag cultures in brain heart infusion (Songer et al., 2007). An aliquot (1.25 ml) of solution, containing 2 x 10^6 heat-shocked NTCD spores, was administered intragastrically, followed by 20 ml of milk replacement.

Lactobacillus sp.

The *Lactobacillus* sp. preparation was prepared as follows: 75 billion CFUs of Probiotic Complex (GNC, General Nutrition Corporation, Pittsburg, PA) were used to fortify the amount of *Lactobacillus acidophilus* in yogurt. One capsule containing 75 billion CFUs of probiotic was thoroughly mixed with 190 ml of yogurt. Each piglet was administered 5 ml of this fortified yogurt to provide an approximate total amount of 2 x 10^6 *Lactobacillus* sp.

Inoculation

Approximately four hours after birth, groups 2, 3, 5, and 6 received either NTCD or *Lactobacillus* sp. intragastrically using an 8 French catheter as an oral-gastric tube (Sovereign TM, Tyco/Healthcare, Mansfield, MA). At 20 hours after birth (16 hours post-prevention intervention), all challenged pigs received a 1.25 ml of inoculum preparation

containing heat-shocked toxigenic *C. difficile* spore solution intragastrically as previously described (Arruda et al., 2013).

Necropsy

Clinical scores were independently and blindly scored for all pigs at the completion of study. Piglets were monitored for 72 h post-challenge and then euthanized by an intravenous overdose of pentobarbital. Gross observations at necropsy included 1) body condition (normal, thin, emaciated), 2) hydration status (normal, mild, moderate, severe dehydration), 3) perineal fecal staining (none, mild, moderate, severe), 4) consistency of colonic contents (-1 = firm/pelleted, 0 = normal, 1 = pudding-like, 2 = watery) , 5) mesocolonic edema (mild = 1 mm separation between loops, moderate = 2–3 mm separation between loops, severe >3 mm separation between loops), and 6) the presence of visible colonic luminal necrosis. All were scored independently in a blinded fashion as previously described (Yaeger et al., 2007; Lizer et al., 2013; Arruda et al., 2013). Necropsies, clinical sign scores and gross lesion scores were completed by the same two individuals for all experiments (PHEA and DMM).

Sample collection

Rectal swabs were taken from all pigs prior to inoculation. At necropsy, fresh and formalin fixed tissues were collected with instruments disinfected between animal necropsies. Samples included: ileum, jejunum, descending colon, cecum, and a cross section of spiral colon containing 4-5 loops. Colonic and cecal contents were collected and stored in sterile plastic cups. An ileal swab (Dacron® Fiber Tipped, Fisher brand®, Leicestershire, UK) was also taken at necropsy.

Toxin detection

Rectal swabs collected prior to inoculation and pooled colon and cecal contents (from same pig) collected at necropsy were assayed for *C. difficile* toxins. A commercially available toxin ELISA kit (C. DIFFICILE TOX A/B II^{TM}, Blacksburg, VA) was used to semi-quantitatively measure the amounts of toxin from 0 (no toxin detection) to 4+ (marked toxin detection) as indicated by the manufacturer. Swabs and intestinal pooled content were frozen and stored at -80 °C until completion of study. All samples were processed simultaneously and in accordance with manufacturer instructions and analyzed on a microplate reader (IDEXX Corp, Molecular Device, Lake Forest, IL).

Histopathology

Tissue sections were collected in 10% neutral buffered formalin. Sections were submitted for routine tissue sectioning followed by paraffin embedding and staining with hematoxylin and eosin. All tissues were examined by a veterinary pathologist (PHEA) who was blinded to animal group designation. Large intestinal sections were assessed for goblet cell loss, neutrophilic aggregates within the lamina propria, and mucosal epithelial defects as previously described (Lizer et al., 2013; Arruda et al., 2013). See Table 2 for detailed microscopic scores.

Scoring

Four categories of scores were compared: 1) clinical signs, 2) ELISA results 3) mesocolonic edema, and 4) microscopic lesions. Summing the scores for body condition, hydration status, and perineal staining were done to create clinical signs scores. ELISA assays were performed on fecal and colon contents at the beginning and end of the experiment. At necropsy, pathologists that were blinded to the treatment group and assigned the mesocolonic edema score. Microscopic lesion score was the sum of scores for all

histopathology categories. Score system has been previously described (Lizer et al., 2013; Arruda et al., 2013).

Statistical methods

Scores for clinical signs, gross and microscopic lesions were analyzed by a non-parametric test. Kruskal-Wallis test was used to determine if there was an overall difference among study groups. Pair-wise comparison was performed using Wilcoxon test; (*p*) value were then adjusted using Bonferroni correction. Correlations between microscopic lesions, mesocolonic edema, and ELISA results were accessed by Spearman's rank correlation coefficient, a non-parametric test. JMP 9 (JMP®, Cary, NC) statistical software was used to perform analyses.

Results

Bacterial Culture

C. perfringens and E. coli were isolated from majority of piglets regardless study group. Nine isolates were randomly selected for PCR genotype. 100% of *C. perfringens* isolated were determined to be type A.

No hemolytic *E. coli* was isolated; PCR genotype was performed on nine randomly selected isolates. One isolate was positive for STb toxin gene; however, all were all negative for pilus antigen and other associated toxin genes. Salmonella was not isolated from any intestinal swab.

Clinical signs

Clinical scores were independently and blindly scored for all pigs at completion of the study. Overall statistical evaluation of clinical signs scores from study groups revealed no statistical difference ($p>0.05$).

The majority of pigs at necropsy from GROUPS 1, 2 and 3 presented with normal body condition as well as hydration status: 90% (9 out of 10 pigs), 85% (11 out of 13 pigs) and 93% (13out of 14 pigs), respectively. Among Groups receiving the toxigenic isolate (GROUPS 4, 5 and 6) normal body condition and hydration status accounted for only 66% (23out of 35 pigs), 61% (21out of 34 pigs), 61% (27out of 44 pigs) respectively; although this difference was not statistically significant ($p>0.05$). Pigs receiving the toxigenic isolate (GROUPS 4, 5 and 6) had slightly higher clinical scores when compared to GROUPS 1, 2 and 3 having mild diarrhea and some degree of dehydration; 23% of pigs receiving the toxigenic strain presented some level of dehydration associated with loss in body condition while only approximately six percent of pigs from group 1, 2 and 3 showed similar levels of dehydration and clinical signs.

Gross lesions

GROUP 5 presented significant lower scores when compared to GROUPS 4 and 6 ($p=0.01$). Other group comparisons failed to achieve statistical significance. Grossly visible mucosal necrosis was not observed within the cecum or spiral colon of individual piglets in all experiments. Figure 1 demonstrates the individual data points and median across different study groups.

All animals were fecal toxin ELISA negative at the beginning of the experiment. At necropsy (72 hours post inoculation), ELISA results showed that GROUP 5 had lower levels of toxin when compared to GROUPS 3, 4 and 6 (Figure 2). Additionally, the prevalence of positive samples was markedly different among these groups; toxins were detected in only 5.8% of pigs (2 out of 34 pigs) from GROUP 5 while toxins were detected in 35.7% (group 3), 25.7% (group 4) and 28.8% (group 6).

Microscopic lesions

Histopathologic examination revealed classic microscopic large intestinal lesions characterized by variable numbers of neutrophils within lamina propria, a loss of goblet cells and single to multiple sites of epithelial erosion or ulceration which were occasionally covered by moderate amounts of cellular and karyorrhectic debris and fibrin; lesions were only observed within colon and cecum (See Figure 3. for Group lesions distribution score). Statistical comparison among groups showed that GROUP 4 had higher scores when compared to other groups. GROUP 5 presented lower microscopic scores when compared to GROUPS 3, 4 and 6; GROUP 5 scores were markedly similar of piglets belonging to GROUPS 1 and 2. Although numerical differences were observed, pair comparisons did not yield significant results

Correlations

Associations among mesocolonic edema, toxin levels and microscopic lesions were accessed and results showed that mesocolonic edema, ELISA toxin levels and microscopic lesions are highly and significantly correlated among each other with consistent (p) values < 0.001 on all combinations

Discussion

Clostridium difficile disease is the most important cause of nosocomial diarrhea in humans; the disease has become a major healthcare concern as incidence and severity of disease have increased significantly over recent years (Kelly and LaMont, 2008; Chumbler et al., 2012). Numerous studies have also documented the susceptibility of the neonatal piglet to such infection; affected piglets commonly develop watery to pasty yellow diarrhea in the first week of life which impact growth and increases variation among litter mates (Songer, 2004).

Despite the significant health and economic impact of disease in humans and other species, no commercial vaccine is available. Once diagnosed in humans, the disease is commonly treated by discontinuing antibiotic use (Poutanen and Simor, 2004; Keessen et al., 2011) and administration of probiotic bacteria and treatment with metronidazole or vancomycin (Parkes et al., 2009; Fitzpatrick, 2013). Most recently a technique known as fecal transplant is gaining acceptance and importance on the treatment of disease in humans (Vaishnavi, 2014).

Different studies have shown that the normal gastrointestinal flora inhibits *C. difficile* growth and toxin release (Borriello, 1990; Parkes et al., 2009) and, therefore, disruption of normal flora is the major risk factor for *C. difficile* disease development in humans. Probiotics are defined by the World Health Organization as "living organisms, which when administrated in adequate amounts, confer a health benefit to the host" (Parkes et al., 2009). Potential mechanisms of how probiotic benefits the host have been described in different studies (Parkes et al., 2009; Fitzpatrick, 2013; Hell et al., 2013). In summary, the most important mechanisms include competitive exclusion; bacterial metabolic activity; preservation of gut barrier function; influence on water and ion channels; influence of innate nervous system; modulation of signal transduction; and stimulation of innate immune system and induction of adaptive immunity (Hell et al., 2013).

The two types of bacterial probiotics utilized in this study included a non-toxigenic strain of *C. difficile* (NTCD) and a commercially available probiotic composed of *Lactobacillus* sp. Different studies using hamster models have shown that colonization with a NTCD can prevent CDI (Borriello and Barclay, 1985; Seal et al., 1987; Sambol et al., 2002). Another study, this time involving two human patients with recurrent CDI showed significant improvement after treatment with NTCD (Seal et al., 1987). Songer et al have shown that

piglets, in farm settings, exposed to NTCD had lower levels of fecal toxin when compared to controls (2007). To our knowledge this is the first experiment, in controlled settings, performed to investigate the benefits of such an alternative in piglets.

Macroscopic evaluation of colon and cecum wall did not reveal necrotizing lesions; this result is consistent with our previous findings where piglets were challenged with different numbers of spores varying from $2x10^6$ to $2x10^9$ spores/ml and necropsied at 72 hours post inoculation (Arruda et al., 2013). Mesocolonic edema, although not pathognomonic, is still considered the major and often only macroscopic lesion associated with clinical cases of CDI in piglets. Results from this study showed that piglets challenged with toxigenic *C. difficile* are more likely to have mesocolonic edema when compared to control piglets. This result is in accordance with previous literature (Songer et al., 2000). Interestingly, piglets administrated with NTCD then challenged showed significant lower rates of mesocolonic edema when compared to positive control challenged piglets and were not statistically different from control animals; for instance, mesocolonic edema was observed in 54% of pigs from GROUP 4 and only in 17% of pigs from GROUP 5. These results indicate a possible benefit of NTCD on the prevention of the development of macroscopic lesions associated with CDI.

Overall, piglets from control groups presented a trend of lower clinical scores when compared to challenged groups (GROUPS 4, 5 and 6); however, statistical significance was not achieved. The large normal variation in those variables and the short study period of three days might have contributed to the lack of statistically significant findings of this study.

It is known that toxins A and B are the main virulence factors of CDI; the pathophysiology and mechanisms of disease development has been described in different

studies (Davies et al., 2011; Chumbler et al., 2012). Toxin level detection through ELISA assays is commonly utilized as a diagnostic tool across different species; ELISA assays were performed on colonic contents collected at necropsy. The prevalence of pigs tested positive for the toxins was lower among piglets receiving NTCD prior challenge when compared to positive control piglets as well as challenged piglets that also received *Lactobacillus* sp. as probiotic. Only 5.8% of the pigs from GROUP 5 tested positive while toxins were detected in approximately 30% of piglets from GROUPS 3, 4 and 6.

Microscopic lesions observed within challenged pigs include loss of goblet cells, multifocal erosions and ulcerations which occasionally contain variable amounts of fibrin, necrotic debris and degenerate and non-degenerate neutrophils (volcano-like lesion). These microscopic findings are often observed in pigs with CDI. Diarrhea commonly observed is a result of decreased and/or impairment of colonic function.

Histopathologic examination is the most objective and accurate form to characterized and diagnose CDI in piglets. Yaeger et al. (2007) showed that constipation is commonly observed in CDI piglets and diarrhea is an inconsistent finding. Although toxin levels, mesocolonic edema and microscopic lesions were positively correlated on the present study; evaluation of field cases of CDI showed that a large proportion of healthy piglets (79%) were positive for *C. difficile* toxins (Yaeger et al., 2007).

Combination of results achieved from statistical analysis of clinical scores, ELISA results and macroscopic and microscopic lesions suggests a benefit to the administration of NTCD as a competitive exclusions technique to prevent CDI in piglets. Data from this study does not support the use of a single administration of *Lactobacillus* sp. as an alternative to prevent the development of disease.

Multiple studies have investigated the efficacy of probiotic bacteria in the prevention of CDI; however, there still is great controversy among researchers. Recently, one meta-analysis (McFarland, 2006) and two systemic reviews (Dendukuri et al., 2005; Pillai and Nelson, 2008) on this topic have shown enough evidence to support the benefit of using probiotics to prevent CDI in humans (Parkes et al., 2009). Probiotics in humans are administrated at multiple time points, as these bacteria often do not permanently colonize the intestines and will completely disappear in about 5-7 days (Mecenier et al., 2000). In this present study, the probiotic was administrated as a single dose at the beginning of the study. Our research group hypothesizes that this might have influenced the results obtained. The single administration was chosen based on the logistic challenges associated with multiple administrations of the product in a farm setting. Study pigs were manipulated individually and intragastrically administered with the probiotic product; this procedure was labor-intensive and likely difficult to mimic on commercial farms due to large numbers of pigs and limited farm personal. Development of new equipment with self-feed capabilities to neonate pigs might allow the use of daily administration of probiotics.

Single administration of NTDC did not produce side effects and was well tolerated by study piglets. Significant reduction in toxin levels, macroscopic and microscopic lesions were observed in piglets administered NTCD prior to challenge. Although the objective of this study was not to elucidate the mechanism by which NTDC prevents or reduces CDI; we hypothesis that competitive exclusion, where non-toxigenic strains will colonize the same niche of toxigenic strains and decrease the amount of bacterial colonization and consequently reduce disease. Other factors including competition for nutrients, modulation of immune response and cross-talk among bacteria via quorum sense are also possibly involved.

Piglets are born with a virtually sterile intestinal tract. However colonization begins at parturition with different species of bacteria including *C. difficile;* Hopman et al showed that nearly one hundred percent of piglets acquire *C. difficile* within 48 hours-of-life (2011). Not all strains of *C. difficile* are toxigenic, strains lacking the toxin A and B genes are considered non-pathogenic. The true prevalence of pigs naturally colonized with NTCD has not yet been investigated. The prevalence of *C. difficile* carriage in humans is estimated to be between 4% and 7.6% where 42 to 50% of the isolates are non-toxigenic (Natarajan et al., 2013). Metagenomic studies investigating how the NTCD strain potentially modulates and alters the dynamic process of bacterial intestinal colonization in the neonate might shed light on the mechanism of protection conferred by this technique.

Conclusion

This study showed that the administration of NTCD decreased prevalence of toxin-positive piglets, reduce mesocolonic edema and histopathologic lesions when compared to GROUPs 4 and 6 (*C. difficile* challenge piglets). Our research group believes that a treatment regime using only NTCD might not be the silver bullet veterinarians and producers have been looking for; however, a multicomponent approach involving good hygiene practices (decrease of exposure), decreased environmental stress and good management practices in conjunction with administration of a non-toxigenic strain of *C. difficile* may significantly prevent or ameliorate CDI in neonate piglets.

Conflict of interest

None of the authors had a personal or financial conflict of interest

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Tables and Figures

Table 1. Experimental design for 1 day-old piglets administered with different bacterial probiotics and subsequent challenge with toxigenic *C. difficile* isolate ISU-15454-1, ribotype 078

Group	n*	Treatment¶	Inoculation ł	Challenge Dose
1	10	-	No	-
2	13	Non-toxigenic *C. difficile* spores @ 2 x10^6	No	-
3	14	*Lactobacillus spp.*/Yogurt	No	-
4	35	-	Yes	*C. difficile* spores @ 2 x10^6
5	34	Non-toxigenic *C. difficile* spores @ 2 x10^6	Yes	*C. difficile* spores @ 2 x10^6
6	44	*Lactobacillus spp.*/Yogurt	Yes	*C. difficile* spores @ 2 x10^6

* Number of cesarean derived piglets per group.
¶ Treatment was administered at 4 hours after parturition.
ł Piglets were challenged with a toxigenic *C. difficile* strain 16 hours post-probiotic administration.

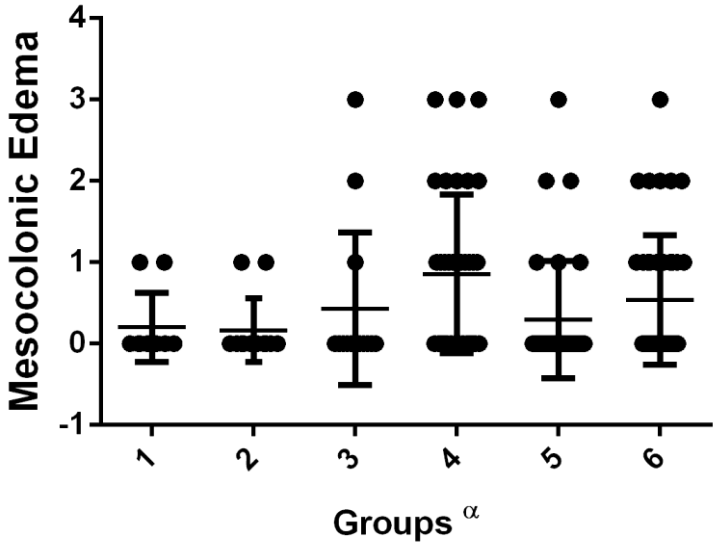

Figure 1. Mesocolonic edema scores according to study groups including 1 day-old piglets administered with different bacterial probiotics and subsequent challenged with toxigenic *C. difficile* isolate ISU-15454-1, ribotype 078

α GROUP 1 negative control, GROUP 2 non-toxigenic *C. difficile* (NTCD) only, GROUP 3 *Lactobacillus* sp. only, GROUP 4 toxigenic *C. difficile* strain only, GROUP 5 NTCD and challenged with the toxigenic *C. difficile* strain, GROUP 6 *Lactobacillus* sp. and challenged with the toxigenic *C. difficile* strain.

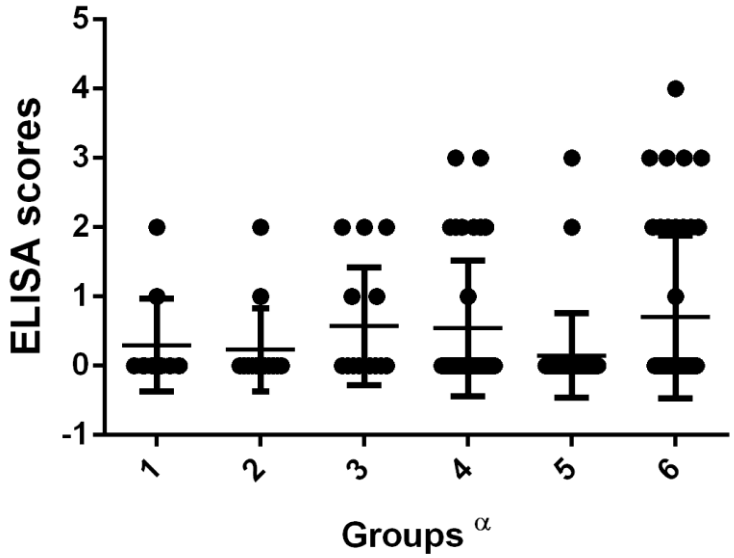

Figure 2. *Clostridium difficile* toxin A/B ELISA scores of 1 day-old piglets (study animals were divided on 6 different groups [α]) administered with different bacterial probiotics and subsequent challenge with toxigenic *C. difficile* isolate ISU-15454-1, ribotype 078.

α GROUP 1 negative control, GROUP 2 non-toxigenic *C. difficile* (NTCD) only, GROUP 3 *Lactobacillus* sp. only, GROUP 4 toxigenic *C. difficile* strain only, GROUP 5 NTCD and challenged with the toxigenic *C. difficile* strain, GROUP 6 *Lactobacillus* sp. and challenged with the toxigenic *C. difficile* strain.

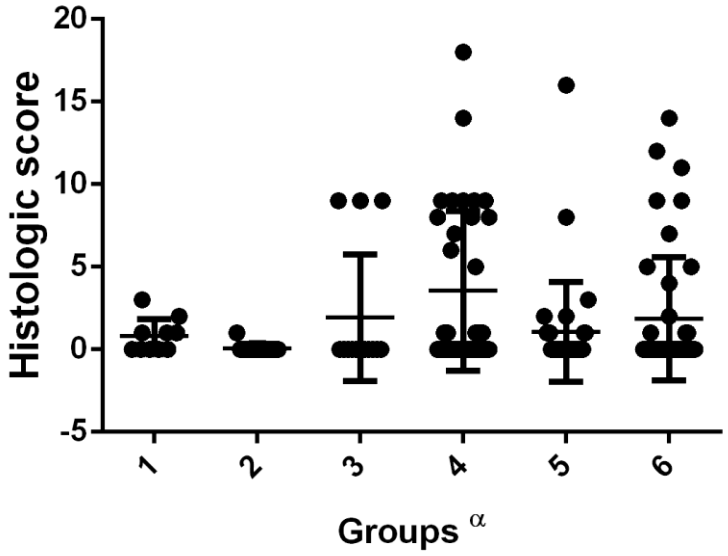

Figure 3. *Clostridium difficile* histopathologic scores of 1 day-old piglets (study animals were divided on 6 different groups [α]) administered with different bacterial probiotics and subsequent challenge with toxigenic *C. difficile* isolate ISU-15454-1, ribotype 078.

α GROUP 1 negative control, GROUP 2 non-toxigenic *C. difficile* (NTCD) only, GROUP 3 *Lactobacillus* sp. only, GROUP 4 toxigenic *C. difficile* strain only, GROUP 5 NTCD and challenged with the toxigenic *C. difficile* strain, GROUP 6 *Lactobacillus* sp. and challenged with the toxigenic *C. difficile* strain

CHAPTER 4. DEVELOPMENT OF A PORCINE LIGATED INTESTINAL-LOOP MODEL TO IVESTIGATE THE PATHOGENESIS OF *CLOSTRIDIUM DIFFICILE* TOXINS A AND B

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Abstract

Toxins A and B (TcdA and TcdB, respectively) are the major virulence factors associated with *Clostridium difficile* disease. Despite the international importance of the disease, there still exists a great deal of controversy regarding the role of TcdA and TcdB in the pathogenesis and immune response in vivo. Therefore, the objective of this pilot study was to develop a pig gut-loop ligation model to study the pathophysiology of TcdA and TcdB independently, and in combination, in segments of small intestine and colon. Eight, seven day-old conventionally-reared piglets were anesthetized and intestinal loops within jejunum, ileum and colon were surgically constructed. Each loop received either three ml of PBS (control), TcdA (204.8 ng/ml), TcdB (320 ng/ml) or combination of both toxins (102.4ng/ml and 160ng/ml, respectively). Results of this pilot study show histologic lesions were primarily localized within small intestine segments while colon segments were unremarkable. Once administrated, TcdA and TcdB simultaneously produced more severe histologic lesions when compared to individual toxin administration, suggesting a synergism between the two toxins. Additionally, TcdA exposure resulted in slightly higher histopathologic lesions when compared to TcdB alone. Relative gene expression including CASP1, HAMP, ITGB2, CSF3, IL17A, IL1B, IL8, NOS2, TLR4 and TNFA were analyzed

within intestinal loops; however, no association between toxin(s) exposure and expression was found. We believe this model has the potential to contribute to investigations into pathogenesis of *Clostridium difficile* infection.

Introduction

Clostridium difficile, a Gram positive, anaerobic, spore-forming bacterium, is a major health concern for hospitalized patients. It is the most common pathogen associated with antibiotic-associated colitis and leading cause of infectious diarrhea in hospitals worldwide (Chumbler *et al.*, 2012; Kelly and LaMont, 2008). Disease is not limited to humans and has been described in several other species including non-human primates, pigs, horses, primates, rabbits, rats, dogs and cats (Arroyo *et al.*, 2005; Debast *et al.*, 2009; Hopman *et al.*, 2011; Keessen *et al.*, 2011; Norman *et al.*, 2009).

Disruption of the gastrointestinal flora by antimicrobial usage is recognized as one of the most important risk factors of the disease (Lawley *et al.*, 2009; Rupnik *et al.*, 2009). Clinical signs associated with *C. difficile* infection (CDI) commonly include fever, severe abdominal pain and diarrhea; histopathologic examination of clinical cases often reveals pseudomembranous colitis. Recently, morbidity and mortality in humans from CDI has dramatically increased as a result of antibiotic usage patterns, an increase in community acquired infections and the emergence of specific strains that appear to be more virulent. (Carter *et al.*, 2010).

Despite the recent characterization of numerous surface layer proteins associated with CDI, it is well accepted that the toxins A and B are the major virulence factors associated with disease development (Borriello, 1998; Carter *et al.*, 2010; Davies *et al.*, 2011; Modi *et al.*, 2011; Voth and Ballard, 2005). Some strains of *C. difficile* also produce an ADP-

ribosylating binary toxin; however, the role of this toxin in the pathogenesis of disease development has not yet been elucidated (Davies *et al.*, 2011).

Previous work in hamster suggested that TcdA alone can induce most of the clinical signs and pathology associated with CDI, and that TcdB was not toxic to animals unless TcdA was administered simultaneously (Lyerly *et al.*, 1985; Voth and Ballard, 2005). However, recent studies and the appearance of the 027 ribotype strain have challenged this theory. The use of genetically modified strains of *C. difficile* via truncation and subsequent inactivation of particular toxin genes have allowed the investigation of each specific toxin`s role in hamster and in vitro tissue culture models. Results from these studies show that TcdB, not TcdA, is essential for disease development, and disruption of TcdB genes significantly reduced the virulence phenotype (Lyras *et al.*, 2009). Clearly, the importance of TcdA and TcdB in disease pathogenesis is controversial and additional studies to better characterize the role of each toxin are warranted.

The objective of this study was to develop a gut-loop ligation model using swine to study the pathophysiology of TcdA and TcdB independently and in combination in different segments of small intestine and colon. We believe that an understanding of the toxins` effects, the associated immunologic response, and intestinal gene expression are essential for the development of chemotherapeutics and vaccine development for the treatment and prevention of CDI.

Materials and Methods

Animals and experimental procedures

Eight conventionally-reared, Reproductive and Respiratory Syndrome Virus negative, 7 days-old piglets were obtained from a commercial sow farm. Piglets were kept in a single

2x2 m pen at a BSL-2 animal facility at Iowa State University (ISU) prior to the intestinal ligation surgery and fed a mixture of commercial puppy milk replacement (ESBILAC, Hampshire, IL) and yogurt (Great Value®, Walmart, Bentonville, AR) *ad libitum.* Piglets were fasted for 4 hours prior to surgery and rectal swabs were collected for toxin ELISA testing.

A pre-surgical dose of butorphanol (0.2-0.4 mg/kg IM) was administered prior to anesthetic induction. Piglets were mask induced with isoflurane followed by tracheal intubation and maintained with isoflurane during the procedure. An IV catheter was placed after induction and piglets were positioned in dorsal recumbency for surgery. Following aseptic preparation of the surgical site, a subcutaneous infusion of lidocaine on the ventral midline was administered along with a dose of buprenorphine (0.01-0.02 mg/kg IM or IV) to minimize discomfort associated with the 8 cm laparotomy incision. Three 15 cm segments of small intestine were exteriorized with approximately 10 cm loops constructed. Anatomical locations of the loops included 1) proximal jejunum 2) distal (aboral) jejunum and 3) ileum. Additionally, one loop of similar length from the centripetal coils of the ascending colon was exteriorized and ligated. Three ml of solution containing either TcdA, TcdB, TcdA and TcdB or PBS were administrated intraluminally using a 25 gauge needle according to the experimental design (Table 1). Intestinal loop-construction and toxin administration are illustrated in Figure 1. The loops were then returned to the abdominal cavity. The abdominal incision was routinely closed and piglets recovered uneventfully within approximately 20 minutes. Postoperatively, piglets were individually housed in 18 gallon plastic totes (Rubbermaid®, Port Washington, NY) for 8 hours and monitored every 30 minutes for any clinical discomfort prior to euthanasia. Eight hours postoperatively blood samples were

collected and piglets were humanly euthanized by an intravenous overdose of pentobarbital. The experimental protocol was approved by the ISU Institutional Animal Care and Use Committee.

C. difficile toxin A and Toxin B

Commercially available purified TcdA and TcdB (The Native Antigen Company, Oxfordshire, UK) were serially diluted in a PBS solution. Dilutions were subsequently tested using a toxin-specific ELISA according to manufacturer's recommendations (TechLab, Blacksburg, VA) to determine the approximate concentration of toxin equivalent to a 3+ result on a scale of 0 to 4+ (data not shown). A 3+ result was selected due to the majority of *C. difficile* disease in piglets, where toxin can be detected, range from 2+ to 4+ on this test (ISU Veterinary Diagnostic Laboratory). 204.8 ng/ml of TcdA and 320 ng/ml of TcdB were administered in the designated loops according to experimental design (see Table 1.). Loops administered both TcdA and TcdB received 102.4ng/ml and 160ng/ml, respectively. A total of 3 ml of PBS and toxin(s) was injected into each loop.

Gene expression

At necropsy, approximately 5 cm segments of each intestinal ligated-loop were collected and serially sliced in sections of 0.5 cm and immediately placed on RNA collection buffer (RNAlater®, Life technologies, Carlsbad, CA) and frozen at -80 until RNA isolation was performed. Approximately 150 mg of tissue was homogenized in 1.4 ml TriReagent (Life Technologies) in a M-tube using a GentleMACS™ dissociator (Miltenyi Biotec, San Diego, CA). RNA was extracted from 0.35 ml of the homogenate using the MagMAX™-96 for Microarrays Total RNA Isolation Kit according to the manufacturer's (Life Technologies) protocol with minor modifications including DNAse treatment of the sample

with the spin isolation protocol. Following RNA extraction, cDNA was synthesized using SuperScript® VILO Master Mix according to manufacturer's recommendations (Life Technologies). Real-time PCR was performed on a 7900 real-time instrument with TaqMan® primers and probe specific for swine mRNA targets with TaqMan® master mix (Life Technologies, Carlsbad, CA). Genes analyzed include: CASP1 –caspase 1; HAMP - Hepcidin antimicrobial peptide; ITGB2- integrin, beta 2 (complement component 3 receptor 3 and 4 subunit); CSF3 - colony stimulating factor 3 (granulocyte); IL17A -interleukin 17A; IL1B - interleukin 1, beta; IL8 interleukin 8; NOS2 - nitric oxide synthase 2, inducible; TLR4 - toll-like receptor 4; TNF alpha - tumor necrosis factor (TNF superfamily, member 2). All samples were run in duplicate and data analyzed using the 2-ddCt method (Livak and Schmittgen, 2001)

Histopathologic examination

Tissue sections were collected in 10% neutral buffered formalin and allowed to fix for at least 48 hr. Sections were then submitted for routine tissue sectioning followed by paraffin embedding, cutting (4 µm) and staining with hematoxylin and eosin. All tissues were examined by a veterinary pathologist (PA) blinded to piglet and section identification. Small and large intestinal sections were assessed and scored for goblet cell loss, neutrophilic aggregates within the lamina propria, and mucosal epithelial defects as previously described (Arruda *et al.*, 2013; Lizer *et al.*, 2012).

Results

Gene expression results

For all intestinal samples collected, mRNA levels of BACT, CASP1, HAMP, ITGB2, CSF3, IL17A, IL1B, IL8, NOS2, TLR4 and TNFA were analyzed using real-time PCR. Data

is expressed as the fold change in gene expression in loops collected from toxin-treated piglets relative to anatomically similar loops from control pigs receiving PBS (Table 2). Results were calculated using the 2-ddCt method (Livak and Schmittgen, 2001) such that a value of 1 represents no change in expression, a value > 1 represents the fold increase and a value < 1 represents a fold decrease relative to the same respective section from loops treated with PBS (control pigs). Relative gene expression from jejunal loops revealed an increase in mRNA expression of HAMP, IL17A and IL-8; however, these results were primarily associated with piglet C (TcdA and TcdB) and piglet E (TcdA). There was marked variation in gene expression levels between piglets.

Results from ileal loops revealed a consistent increase in all genes from piglet E (TcdA) and piglet H (TcdB). Interestingly, gene expression from other challenged piglets was down regulated when compared to control piglets. Analysis from colon samples revealed a consistent down regulation of selected genes among challenged piglets.

For the jejunal loops, each pig had one loop that was only administered PBS. Data analysis was performed for those piglets by the method described above (relative to control pigs in which PBS was administered in all loops). In addition, the data was analyzed as the relative gene expression of a toxin-administered jejunal loop compared to a PBS-administered jejunal loop segment within the same piglet (Table 3). Making comparisons within the same piglet showed there was a marked increase in the relative expression of HAMP, ITGB2, CSF3, IL17A, IL1B, IL8, NOS2, TLR4 and TNF in piglet C in which one loop received TcdA and TcdB and other loop received PBS. These results are consistent when data was compared relative to loops from pigs that were given only PBS; though additional genes were increased as well.

Histopathologic results

Microscopic examination of intestinal sections from control piglets (A and B) did not reveal significant lesions. Goblet cells were preserved throughout all the segments of small and large intestines and mucosal architecture was unremarkable. Lamina propria within proximal jejunum of piglet B was variably infiltrated by small aggregates of neutrophils. No other lesions were observed. Intestinal sections receiving both TcdA and TcdB (distal jejunum and ileum from piglets C and D) and TcdA alone had mild to moderate lesions predominately characterized by multifocal to locally extensive infiltration of neutrophils, which were often associated with scant amounts of cellular debris and goblet cell loss. The mucosa was occasionally disrupted and eroded; however, this change was not a predominant finding in the sections. Proximal jejunum sections from piglets C and D (PBS controls) were unremarkable; however, occasionally rare neutrophils were observed within lamina propria.

Sections of proximal jejunum administrated TcdB were unremarkable; however, ileal sections from both piglets receiving TcdB showed mild lesions characterized by variably sized aggregates of neutrophils expanding the lamina propria and occasionally disrupting the epithelial architecture (exocytosis). No ulcerations or erosions were observed throughout examined sections.

Histologic examination of large intestinal from all piglets did not reveal significant lesions. A detailed summary of histologic scores is presented in Table 4.

Discussion

Toxins A and B (TcdA and TcdB, respectively) are the major virulence factors associated with CDI; however, the exact mechanisms by which they elicit such gross and histologic lesions and the associated-immunological response are not yet completely understood. TcdA

and TcdB are part of the large clostridia glucosylating toxin family; they are very large toxins with molecular masses of 308 and 250 kDa, respectively. Both TcdA and TcdB are internalized via receptor-mediated endocytosis. A decrease in the pH within the endosome compartment results in toxin conformation changes and pore formation allowing the release of the enzymatic domain of the toxin into the cytosol. Once within the cytosol of the cell, TcdA and TcdB target Rho GTPases which include Rho, Ras and Cdc42. Inactivation of these cellular molecules disrupts cell signaling and compromises actin cytoskeleton regulation, which eventually leads to cell rounding and death (Chumbler *et al.,* 2012; Davies *et al.,* 2011; Dillon *et al.,* 1995; Jank and Aktories, 2008; Just *et al.,* 1994; Keel and Songer, 2007).

For over 30 years, animal models for *C. difficile* infection have undergone development and are currently being optimized (Best *et al.,* 2012). Several different animals have been used as models to study CDI; some examples include hamster, mice, guinea pig, rat, prairie dogs, quails, rabbits and pigs (Best *et al.,* 2012; Lawley and Young, 2013). The hamster model is the most well described and the most common animal model used to study CDI.

Despite the characterization of this model, hamsters are exquisitely sensitive to the toxins' effects (after antibiotic administration) and commonly die within a few days (Best *et al.,* 2012; Keel and Songer, 2006). Rapid and uniformly fatal disease (Best *et al.,* 2012; Keel and Songer, 2006; Lyerly *et al.,* 1985) is not characteristic of CDI in humans. The rapid progression of disease in this species also poses obstacles when investigating possible treatments and/or preventive options.

CDI is a naturally occurring disease in 1-7 days-old-piglets and considered one of the most common causes of colitis (Debast *et al.,* 2009; Songer, 2004; Yaeger *et al.,* 2007). Pigs

are moderately sensitive to the toxins' effects and severity of clinical signs and histologic lesions can be altered by piglet-age and challenge dose (Steele *et al.,* 2010). Additionally, the marked similarities in nature and progression of CDI in pigs to human disease (Best *et al.,* 2012; Steele *et al.,* 2010) and the wide range of assays and immunoreagents commercially available make the pig model an attractive alternative to study the disease.

The primary objective of this study was to develop an *in vivo* model to investigate the role of TcdA A and TcdB in and the immunologic-profile associated with CDI. Lesions associated with *C. difficile* infection are primarily found in the large intestine and rarely observed in the small intestine. For this study, intestinal-loops included three segments of small intestine and one segment of large intestine. Interestingly, the majority of lesions observed were localized in the small intestinal segments administrated TcdA and TcdB, and TcdA with large intestine segments being consistently unremarkable throughout all piglets.

The following three mechanisms might have influenced the lack of lesions within large intestine sections. First, we hypothesize that this finding may be due to the surgical protocol where, in order to preserve intestinal architectural integrity, intraluminal contents were left undisturbed. This might become relevant for toxin endocytosis as the contents within the small intestinal sections were liquid and markedly different when compared to the pasty-solid content within the colon. Direct contact and time-exposure between toxin and epithelium surface is likely necessary as these toxins are large molecules which are internalized via receptor-mediated endocytosis through the binding of the carboxy-terminal domain to cell carbohydrate receptors (Chumbler *et al.,* 2012; Davies *et al.,* 2011; Keel and Songer, 2007).

Low to no amount of toxin internalization might be associated with the lack of lesions on large intestine sections. Second, purified toxin, not *C. difficile* spores or vegetative bacteria, were administered into intestinal loops. Perhaps the use of the spores or vegetative bacteria instead of purified toxin would yield more severe lesions. The first step of *C. difficile* infection likely consists of attachment and colonization of the intestines. A previous study by Gomez-Trevino *et al*. showed that *C. difficile* is capable of attaching to different cell lines *in vitro* (Caco-2 and Vero cells) and *in vivo* (Gomez-Trevino *et al.*, 1996). Colonization allows the bacteria to have direct contact with the cell surface as well as resist some innate immune mechanisms, such as peristaltic movements. It is known that surface layer proteins of the *C. difficile* wall are directly involved in adherence to mucus and epithelium cells (Calabi and Fairweather, 2002). Studies investigating the constituents of *C. difficile* cell wall have identified different surface layer proteins such as Cpw66 (Waligora *et al.,* 2001), GroEL (Ternan *et al.,* 2012), Fbp68 (Barketi-Klai *et al.,* 2011; Hennequin *et al.,* 2003), Cwp84 and the presence of flagella (Tasteyre *et al.,* 2001). In summary, studies have shown that these different proteins are directly related to bacterial colonization and indirectly related to toxin binding and internalization.

It is a consensus among the medical community that disruption of the microbiota is the major risk factor associated with CDI. Microbial population and diversity increases gradually when moving from stomach to small intestines to colon where bacteria densities can reach as high as 10^{12} per grams of content (Rossi *et al.,* 2013). Studies evaluating the impact of the microbial flora have shown that these normal bacterial residents can impact *C. difficile* colonization and in some circumstances toxin binding (Fitzpatrick, 2013; Hell *et al.,* 2013; Parkes *et al.,* 2009). For instance, numerous studies have shown that *Saccharomyces*

boulardii can inhibit TcdA receptor binding through the release of proteases that digest both the toxin as well as the TcdA cellular receptors (Castagliuolo *et al.,* 1996; Pothoulakis *et al.*, 1993; Pothoulakis, 2009). The impact of the loop formation on the intestinal resident microflora was not evaluated in this study and could have played a role in toxin binding, internalization and may be associated with the lack of lesions within the colon segments.

Small intestinal segments administrated both TcdA and TcdB concurrently had higher histologic scores followed by segments receiving only TcdA. Our results are consistent with previous studies suggesting the synergistic effects of these toxins (Du and Alfa, 2004; Kim *et al.*, 1987; Lyerly *et al.*, 1985; Voth and Ballard, 2005). However, TcdA alone resulted in higher histologic scores when compared to TcdB only, which is inconsistent with the most recent literature that states that TcdB is significantly more toxigenic than TcdA (Lyras *et al.*, 2009; Riegler *et al.*, 1995). This discord may be due to the duration of this present study which may have resulted in insufficient time to develop pathology associated with TcdB. He and others (He *et al.*, 2002) have shown that TcdA localizes in the mitochondria as early as 5 to 10 minutes after cell-membrane binding resulting in increased reactive oxygen intermediates, decreased ATP production, release of cytochrome C and activation of the NF-kB pathway that ultimately induces cytokine production (such as IL-8). Release of cytochrome C is associated with initiation of apoptosis (Cai and Jones, 1998; Petit *et al.*, 1996; Susin *et al.*, 1999). Although a majority of cell damage and histologic lesions associated with *C. difficile* are attributed to the glucosylation and inactivation of the GTPases of the Rho family and subsequent cytoskeleton damage, the previous study has shown mitochondrial damage with activation of NF-kB and IL-8 production occurs independently from Rho family inactivation (He *et al.*, 2002). NF-kB activation, in peripheral monocytes

and monocyte cell line, occurs approximately 15 minutes after TcdA has reached the mitochondria and prior to glucosylation of GTPases protein on the cell. Little is known about the time required to elicit the pathologic effects of TcdB. Accordingly, increasing the duration of the study, and therefore the amount of time TcdB is associated with the intestinal epithelium, may be necessary to fully evaluate the effects of TcdB. In addition, slight modifications to the surgical protocol such as removal of intestinal contents prior to inoculum administration along with the longer TcdB period exposure might be beneficial for future studies.

Genes evaluated in this study were selected based on the clinical signs and histologic lesions observed with CDI. Comparison in gene expression from jejunum loops of control and challenged piglets revealed an increase of mRNA levels of HAMP, IL17A, IL-1β, IL-8, NOS2 and TLR4 in piglet C (TcdA and TcdB). Upregulation of these genes was associated with a high histologic score (Figure 2). Histologic examination of the jejunum revealed a moderate to marked neutrophilic infiltration and multifocal goblet cell loss. Evaluation of gene expression between ileum loops from control and challenged piglets revealed a consistent upregulation across examined genes for piglets E (Tcd A) and H (Tcd B). These results are in agreement with histologic scores where moderate to marked neutrophilic infiltration was observed. Interestingly, gene expression from other challenged piglets was slightly down regulated when compared to control piglets; we hypothesized these results might be within normal individual variation and large sample sizes are likely necessary to detect any numerical or statistical difference. Analysis from colon samples revealed a consistent down regulation of selected genes among challenged piglets. These results are in

agreement with histologic findings where no significant histopathologic lesions were observed.

A second method of comparison was performed between different jejunum loops within each piglet. Results from piglet C, where one loop received TcdA and TcdB and another loop which received PBS showed a marked increase of expression of the following genes HAMP, ITGB2, CSF3, IL17A, IL1B, IL8, NOS2, TLR4 and TNF. This result is reflected by the high histologic score observed in jejunum sections administrated TcdA and TcdB when compared with sections receiving PBS.

The main objective of gene expression evaluation was to demonstrate the capability and usefulness of such techniques when investigating the pathophysiology of an infectious disease and the associated inflammatory response; in this case with *C. difficile* infection. Within this study, there were several occasions in which gene expression results were not associated with histologic scores. This difference could be for several reasons. First, a very small portion of the intestine was used for RNA extraction and it's possible the cells in that microenvironment were subject to different levels of toxin. Second, we used the entire intestinal section in our analysis as opposed to a mucosal scraping. There may have been an abundance of RNA from cells not affected by the toxin that diluted the changes in affected cells. Future studies would aim to address these differences to further develop the model.

Conclusions

In summary, our results demonstrate that the porcine intestinal loop model has the potential to become a valuable and repeatable resource to further investigate the pathophysiology and associated inflammatory response associated with individual clostridium toxins within different segments of the intestinal tract. According to this

preliminary data it appears that the TcdA and TcdB, when administered simultaneously, are more toxigenic and capable of inducing a more severe inflammatory response.

Conflict of interest

None of the authors had a personal or financial conflict of interest

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Tables and Figures

Table 1. Experimental design. Intestinal loop segments were named according to anatomical location of loop. Jejunum 1 and Jejunum 2 correspond to proximal and distal portions of the jejunum, respectively. One segment of ileum and colon was constructed per piglet. Individual loops received assigned treatment. Piglets A and B were considered negative controls as no toxins were administrated at any point.

Pig ID	*Intestinal Loops/Treatment*			
	Jejunum 1	Jejunum 2	Ileum	Colon
A	.	PBS[*].	PBS	PBS
B	.	PBS.	PBS	PBS
C	PBS	A&B [π]	A&B	A&B
D	PBS	A&B	A&B	A&B
E	A[†]	PBS	A	A&B
F	A	PBS	A	A&B
G	B[‡]	PBS	B	B
H	B	PBS	B	B

[*]Phosphate buffered saline was used as negative control.
[†] *C. difficile* toxin A, total of 3 ml of 204.8 ng/ml solution was administered/loop.
[‡] *C. difficile* toxin B, total of 3 ml of 320 ng/ml solution was administered/loop.
[Π] Combination of Toxins A and B at 102.4ng/ml and 160ng/ml, respectively.

Table 2. Analysis of relative gene expression quantified using Real-Time Quantitative PCR and the $22^{-\Delta\Delta C}{}_{T}$. Values shown are the relative gene expression of collected loops from challenged piglets compared to anatomically similar loops from control piglets receiving PBS. Value =1 represents no change, value >1 for a fold increase, and value <1 for fold decrease in expression relative to piglets A and B (negative control piglets with PBS treated loops).

*Jejunum**										
Pig ID	CASP1	HAMP	ITGB2	GCSF	IL17A	IL1β	IL8	NOS2	TLR4	TNFα
C	0.95	5.65	1.44	1.24	5.90	2.00	2.64	1.84	1.93	1.40
D	0.76	2.03	1.21	1.09	0.16	0.27	0.16	0.89	0.70	1.04
E	1.10	14.27	0.44	1.39	3.44	1.06	3.01	1.36	1.24	0.41
F	2.75	0.47	0.69	1.14	1.71	0.98	0.48	0.77	2.58	0.30
G	1.39	0.25	0.32	0.31	0.21	0.30	1.00	0.40	1.07	0.43
H	3.34	0.14	0.49	0.54	0.42	0.45	0.75	0.38	1.69	0.38
Ileum										
Pig ID	CASP1	HAMP	ITGB2	GCSF	IL17A	IL1 β	IL8	NOS2	TLR4	TNFα
C	0.75	0.24	1.00	0.88	0.77	0.39	0.42	0.52	0.85	0.82
D	1.50	1.00	1.03	0.92	0.50	2.70	0.48	1.34	0.97	1.12
E	1.40	4.76	1.10	1.67	11.35	3.32	3.77	2.41	2.22	1.88
F	1.58	0.25	1.11	1.43	1.17	0.15	0.09	0.95	0.53	0.68
G	1.70	0.96	0.79	0.36	0.48	0.91	1.83	0.95	1.13	0.95
H	1.75	1.90	1.48	1.22	2.84	5.96	2.12	2.57	1.83	1.84
Colon										
Pig ID	CASP1	HAMP	ITGB2	GCSF	IL17A	IL1 β	IL8	NOS2	TLR4	TNFα
C	0.53	0.68	0.35	0.63	1.80	0.52	1.11	0.39	0.74	0.32
D	0.49	0.62	0.32	0.50	0.55	0.22	0.39	0.94	0.20	0.24
E	0.77	1.20	0.31	0.41	1.11	0.17	0.81	1.05	0.22	0.33
F	0.50	4.36	0.36	0.71	1.27	0.46	1.73	0.42	0.39	0.24
G	0.57	0.28	0.33	0.39	0.72	0.27	0.97	1.15	0.34	0.38

CASP1 –caspase 1; HAMP - Hepcidin antimicrobial peptide; ITGB2- integrin, beta 2 (complement component 3 receptor 3 and 4 subunit); CSF3 - colony stimulating factor 3 (granulocyte); IL17A -interleukin 17A; IL1B - interleukin 1, beta; IL8 interleukin 8; NOS2 - nitric oxide synthase 2, inducible; TLR4 - toll-like receptor 4; TNF - tumor necrosis factor (TNF superfamily, member 2).

*Jejunum segments administrated with toxin A and/or toxin B. Jejunum segments administered with PBS were not include on this analysis

Table 3. Analysis of relative gene expression quantified using Real-Time Quantitative PCR and the $2^{-\Delta\Delta C}{}_t$. Values shown are the relative gene expression of jejunum loop administered toxin compared to jejunum loop segment from same piglet administered PBS. Value =1 represents no change, value >1 for a fold increase, and value <1 for fold decrease in expression relative to that piglet's PBS treated loop.

Pig ID	CASP1	HAMP	ITGB2	GCSF	IL17A	IL1B	IL8	NOS2	TLR4	TNFA
C	0.50	40.49	3.74	1.94	10.14	9.68	5.18	10.56	2.43	5.04
D	0.25	0.30	3.18	0.83	0.20	0.37	0.16	0.62	0.86	1.96
E	0.83	6.21	1.08	1.35	6.20	2.27	1.83	1.98	0.95	1.04
F	2.22	0.39	0.42	0.76	1.85	2.14	2.28	0.61	2.10	0.24
G	1.21	0.61	0.71	0.91	0.90	0.93	0.86	0.66	1.68	0.86
H	1.96	0.09	0.38	1.18	0.21	0.10	0.24	0.25	0.54	0.20

CASP1 –caspase 1; HAMP - Hepcidin antimicrobial peptide; ITGB2- integrin, beta 2 (complement component 3 receptor 3 and 4 subunit); CSF3 - colony stimulating factor 3 (granulocyte); IL17A -interleukin 17A; IL1B - interleukin 1, beta; IL8 interleukin 8; NOS2 - nitric oxide synthase 2, inducible; TLR4 - toll-like receptor 4; TNF - tumor necrosis factor (TNF superfamily, member 2).

Table 4. Histologic scores of different sections of small and large intestines.

ID[†]	*Proximal Jejunum*			*Distal Jejunum*			*Ileum*			*Colon*		
	Goblet cell loss	PMNs[*]	Mucosa	Goblet cell loss	PMNs	Mucosa	Goblet cell loss	PMNs	Mucosa	Goblet cell loss	PMNs	Mucosa
A	.	.	.	0	0	0	0	0	0	0	0	0
B	.	.	.	0	2	0	0	0	0	0	0	0
C	0	0	0	2	3	1	1	2	1	0	0	0
D	0	1	0	2	3	1	1	2	0	0	0	0
E	0	1	0	0	1	0	1	3	0	0	0	0
F	2	3	1	0	3	0	0	0	0	0	0	0
G	0	0	0	0	0	0	0	1	0	0	0	0
H	0	0	0	0	0	0	0	2	0	0	0	0

[*]Polymorphnuclear neutrophils; numbers of neutrophils was counting using 400x magnification.
[†] Piglet identification.

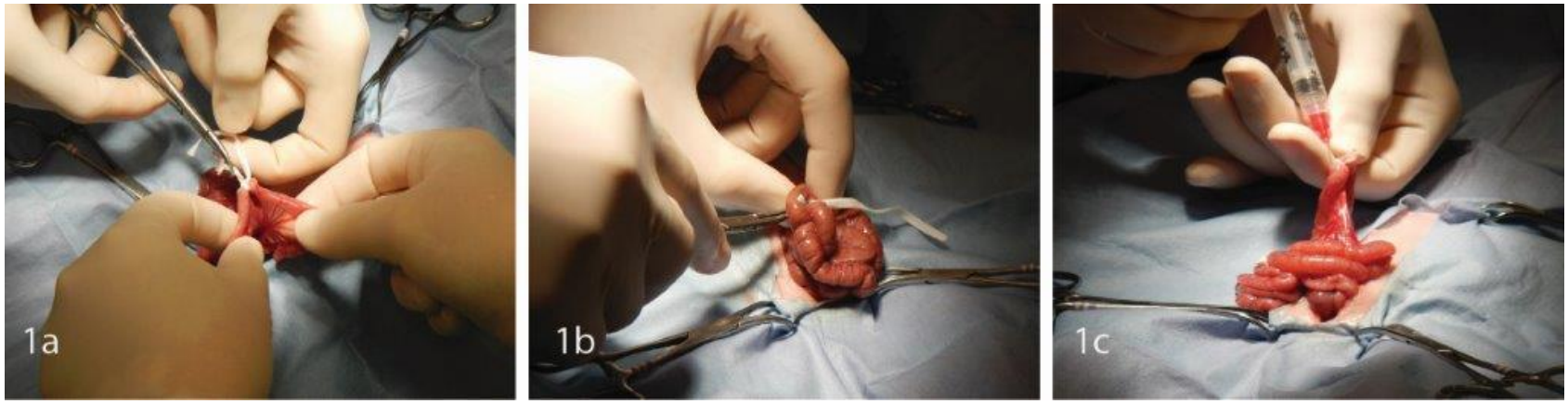

Figure 1. Surgical construction of intestinal loops on 7 day-old anesthetized piglets and intraluminal administration of *C. difficile* TcdA and TcdB.

1a. Construction of loop from proximal jejunum. Loops measure approximately 10 cm in length; 1b. construction of loop from centripetal segment of ascending colon; 1c. administration of 3 ml of solution containing either PBS or TcdA and/or TcdB in PBS on previously constructed loop.

CHAPTER 5. GENERAL CONCLUSIONS

The number of human cases of antibiotic-associated diarrhea (AAD) has increased since the 1950's, which corresponds with the beginning of the antibiotic-era. However, *Clostridium. difficile* was not classified as a pathogen until the late 1970's when a small cohort of patients suffering from AAD was identified based on a common clindamycin administration history (Bartlett J. G. *et al.,* 1977; Tedesco F. J. *et al.,* 1974). The bacterium has become the leading cause of infectious diarrhea in developed countries and the primary cause of AAD worldwide (Bartlett J. G. 2002; Carter G. P. *et al.,* 2010; Keel M. K., Songer, J. G., 2006; Kelly C. P., LaMont, J. T. 2008; McDonald L. C. *et al.,* 2005; Steele J. *et al.,* 2010). The disease is estimated to cost approximately € 3 billion per year in the European Union and between $436million to $3.2 billion annually in the United States.

Clostridium difficile is a Gram positive, anaerobic, spore-forming bacterium commonly found in the environment including soil, water, vegetables (al S. N., Brazier, J. S., 1996), and the gastrointestinal tract of animals (Songer J. G., 1996). *Clostridium difficile* produces metabolically-dormant spores which are directly linked with environmental survival and consequently associated with transmission, epidemiology and pathogenesis of AAD. *Clostridium difficile* spores are highly resistant to oxygen exposure, desiccation, and most common disinfectants, surviving for months and probably years in the environment (Wullt M. *et al.,* 2003). Toxins A and B produced by vegetative bacteria are the major virulence factors; however, the exact mechanisms and the role and importance of each toxin are yet to be fully elucidated.

The disease in humans is often associated with events of intestinal microbiota disruption commonly following antibiotic administration. A similar association is present in

domestic animals; however, immature intestinal flora commonly observed in neonatal animals is also considered a major risk factor to *C. difficile* infection. Human treatment is directed at the reestablishment of the intestinal microbiota by interruption of antibiotic treatment, usage of a probiotic, fecal transplant (Vaishnavi C., 2014) and the use of selective antibiotics. Currently, there is no approved vaccine or antibody therapy available.

Clostridium difficile infection has also been described in several non-human species including pigs, horses, primates, rabbits, rats, dogs and cats (Arroyo L. G. *et al.*, 2005; Debast S. B. *et al.*, 2009; Hopman N. E. *et al.*, 2011; Keessen E. C. *et al.*, 2011a; Norman K. N. *et al.*, 2009). In piglets, CDI often occurs in the first week-of-life. Studies have shown that *C. difficile* intestinal colonization occurs within the first hours of life in the neonatal pig, and nearly all piglets in some herds are colonized within 48 hours of birth (Hopman N. E. *et al.*, 2011). Despite the endemic nature of bacteria, not all piglets develop disease. Reports on affected herds showed that on average 66% of the litters are affected, and within affected litters the morbidity can reach 97 to 100% (Anderson M. A., Songer, J. G., 2008; Songer J. G., 2004). Mortality is highly depended on factors including management and concurrent infectious etiologies. Some studies have reported mortality rates as high as 16% (Anderson M. A., Songer, J. G., 2008). Piglets often showed growth retardation with lower weaning weights averaging a half a kilogram less when compared to unaffected littermates (Songer J. G., 2004). Despite the economic and welfare concerns associate with CDI in piglets, there is a significant lack of knowledge regarding risk factors, pathogenesis and treatment options for CDI in piglets.

This thesis documents our efforts to increase the understanding of risk factors, treatment options and the pathogenesis of CDI in piglets. The first three studies had the

primarily objective to investigate three potential risk factors associated with CDI in piglets; to our knowledge these were the first studies to investigate the impact of bacterial dosage, piglet age and antimicrobial treatments on the development of disease. First, we demonstrated that the *C. difficile* isolate (isolate 15454-1, ribotype 078, toxinotype V) used in this study was able to cause *C. difficile* disease in piglets with development of typical gross and histologic lesions. Results evaluating the role of antibiotic administration as a potential risk factor revealed that the usage of Lincomycin, Ceftiofur, Tylosin or Tulathromycin prior to *C. difficile* challenge did not increase the prevalence of disease or enhance of lesions severity.

We believe that these results can be explained by the fact that one day-old piglets do not have an established intestinal microflora, and therefore antibiotics would not significantly alter the microbiota. Future studies investigating bacterial colonization, succession and competition in the first day-of-life may provide a better understanding of the impact of antibiotic administration on the colonic microbiota.

It is known that the vast majority of CDI cases occur in piglets within the first week-of-life; however, studies investigating the susceptibility of older (10-days-old), commercial piglets to CDI have not been published. Results from this study showed that older piglets are as susceptible to CDI as neonate piglets. However, extrapolation from these results should not be directly applied to field cases as these piglets were raised in a controlled environment considerably different than a commercial farm. Although, these results do demonstrated that 10-days-old piglets have receptors for toxins A and B and, in special circumstances, can develop CDI. Additionally, *C. difficile* dosage was identified as an important risk factor associated with the development of CDI. Increase of bacteria load was associated with

increase of prevalence and severity of microscopic lesions typical of CDI. Therefore, good management practices that decrease the exposure of neonatal piglets to a high challenge dose and allow the establishment of a healthy microflora is likely essential to prevent of CDI in piglets. While antibiotic usage is the most important risk factor for other species, it does not appear to be significantly important in piglets.

Regarding the prevention and control of CDI in piglets; to our knowledge this was the first experiment, under controlled settings, designed to evaluate the benefits of a non-toxigenic *C. difficile* strain and *Lactobacillus* sp. as probiotics in the prevention of CDI in piglets. Results from this study showed that the use of non-toxigenic *C. difficile* (NTCD) as a probiotic may be beneficial; piglets that received NTCD prior to challenge with a toxigenic strain had a decreased prevalence of toxin-positive piglets, less mesocolonic edema and lower histopathologic scores. The use of a single administration of *Lactobacillus sp*. as a probiotic was not beneficial in the prevention of CDI in piglets in this particular study. Future studies investigating the prevalence of NTCD among commercial piglets as well as experiments investigating the impact of NTCD strains on bacterial intestinal colonization in the neonate will likely advance our understanding of the mechanism of protection conferred by the use of NTCD as a probiotic.

Chapter 4 of this dissertation described a pilot study in which a pig intestinal-loop ligation model was developed providing an alternative *in vivo* model to investigate the role of toxins A and B and the cytokine-profile associated with CDI in different segments of the small intestine and colon. In additional to histopathologic characterization of lesions, expression of selected genes was also evaluated. Results from this study showed a potential synergism between toxin A and B; administration of both toxins simultaneously yields higher

histologic scores when compared to individual toxins. These results are in agreement with previous *C. difficile* pathogenesis studies (Du T., Alfa, M. J., 2004; Kim P. H. *et al.,* 1987; Lyerly D. M. *et al.,* 1985; Voth D. E., Ballard, J. D., 2005). Interestingly, toxin A administration led to higher histologic scores when compared to toxin B in this particular study. These latter results are inconsistent with the most recent literature that states that toxin B is significantly more toxigenic than toxin A (Lyras D. *et al.,* 2009; Riegler M. *et al.,* 1995). Genes were upregulated in segments receiving both toxin A and B; however, this finding was inconsistent and not routinely associated with histologic lesions. Surprisingly, a majority of lesions were observed within small intestinal sections while colon was often unremarkable across treatment groups.

When optimized this *in vivo* model has the potential to become a valuable resource, providing a novel and appropriate manner by which to investigate the pathogenic mechanisms of toxins A and B, the associated immunologic response, and intestinal gene expression. We believe that such insight is essential for the development of new antibiotics and vaccine for the treatment and prevention of CDI on humans and other domestic species.

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